

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Feruloyl esterases

Evaluation of their potential for biotechnological applications

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Department of Biology and Biological Engineering

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“Nous ne savons souvent voir que ce que nous sommes prêts à voir. La qualité essentielle du chercheur est son aptitude à admettre qu’il s’est fourvoyé ”

Albert Jacquard

Petite philosophie à l’usage des non-philosophes

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ABSTRACT

Owing to the current efforts to find sustainable alternatives to petrochemical based industries and technologies, enzymatic degradation and valorization of plant biomass has been attracting interest. Due to the complexity of plant biomass, an array of enzymes is required to hydrolyze it, including esterases. Among the esterases involved, feruloyl esterases, which are able to release ferulic acid, were of special interest in this work. Industrial processes aim for enzymes to be as efficient as possible in the designed process conditions, *i.e.* able to perform chemical reactions for as long as possible at the lowest possible cost. Several strategies can be employed to reach these goals, such as (i) finding novel enzymes with the desired properties, (ii) optimizing enzyme production, or (iii) immobilizing enzyme for improved stability or reusability. These strategies were applied in this work to investigate the potential of some feruloyl esterases for industrial applications.

Based on functional annotations, targets originating from microorganisms found in diverse ecological niches were selected. In one study, five putative feruloyl esterases/tannases from two *Aspergillus* fungi were selected. In another study, two multi-domain enzymes displaying two predicted esterase domains from the polysaccharide utilization loci of bacteria in the Bacteroidetes phylum were investigated. The enzymes displayed differences in their preferred reaction conditions (pH, temperature), molecular weights, predicted isoelectric points, as well as substrate preferences. The impact of the production host on the final enzyme properties was investigated in an additional study. We demonstrated that in the case of glycosylated enzymes, careful selection of the production host is crucial for thermostability. Studying immobilization, data showed that the best immobilization yield and the best immobilized enzyme performance were not achieved in the same conditions for any of the enzyme-support couples tested. Investigations of immobilized enzyme transesterification or hydrolysis activities clearly demonstrated that immobilization does affect the catalytic activity of enzymes. In the current status of our knowledge, the way an enzyme is affected by immobilization is not predictable. Increased knowledge about esterase structures, reaction mechanisms and surface properties may however allow such predictions in the future. This thesis contributes to increasing the available information about esterases, and in particular feruloyl esterases.

Keywords: carbohydrate-active enzyme family 1, feruloyl esterase, acetyl esterase, acetyl xylan esterase, enzyme stability, heterologous production, *N*-glycosylation, enzyme immobilization, polysaccharide utilization loci, multi-domain enzyme

Preface

This doctoral thesis partially fulfils the requirements for a PhD degree at the Department of Biology and Biological Engineering, Division of Industrial Biotechnology, Chalmers University of Technology, Sweden. The work presented in this thesis was performed between 2013 and 2019, and was funded by the Swedish Research Council (Vetenskapsrådet, VR Grant 349–2007-8680) via the Linnaeus Centre for Bio-inspired Supramolecular Function and Design–SUPRA. SUPRA is a collaboration program between Chalmers researchers from three departments, Physics, Chemistry and chemical engineering and Biology and biological engineering, focusing on supramolecular systems. The work presented here is part of the “enzyme-catalyzed reactions in confined media” research axis developed in the SUPRA program.

The main part of the work was carried out at the Division of Industrial Biotechnology at Chalmers University of Technology, under the supervision of Professor Pernilla Wittung-Stafshede, Professor Lisbeth Olsson and Dr. Scott Mazurkewich.

Mass spectrometry analysis of the protein glycan was performed in collaboration with Dr. Ekaterina Mirgorodskaya, Proteomics Core Facility, Sahlgrenska Academy, University of Gothenburg, Sweden. The glycoproteomic analysis was supported by the Swedish National Infrastructure for Biological Mass Spectrometry (BioMS), funded by the Swedish Research Council (Vetenskapsrådet).

Cyrielle Bonzom

October, 2019

List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I **Bonzom C**, Schild L, Gustafsson H, Olsson L (2018). Feruloyl esterase immobilization in mesoporous silica particles and characterization in hydrolysis and transesterification. *BMC Biochemistry*, 19 (1). DOI: 10.1186/s12858-018-0091-y

- II **Bonzom C**, Hüttner S, Mirgorodskaya E, Chong S-L, Uthoff S, Steinbüchel A, Verhaert RMD, Olsson L (2019). Glycosylation influences activity, stability and immobilization of the feruloyl esterase 1a from *Myceliophthora thermophila*. *AMB Express*, 9 (126). DOI: 10.1186/s13568-019-0852-z

- III Kmezik C, **Bonzom C**, Olsson L, Mazurkewich S, Larsbrink J (2019). Investigation of multi-domain esterases from soil and gut Bacteroidetes involved in xylan metabolism. *Manuscript in preparation*

- IV **Bonzom C**, Thörn C, Anasontzis G, Schild L, Olsson L (2019). Investigation of five putative esterases from *Aspergillus glaucus* and *Aspergillus zonatus*. *Manuscript in preparation*

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Author's contributions

Paper I: First author. I conceptualized and designed the study, planned and performed some of the enzyme immobilization and enzymatic reactions. I analyzed the data and wrote the manuscript.

Paper II: First author. I conceptualized and designed the study, planned and performed the enzyme production in *Escherichia coli*. I purified, immobilized and carried enzymatic assays on the three enzyme versions. I analyzed the data and wrote the manuscript, with some support for the mass spectrometry analysis.

Paper III: Second author. I planned and performed the production, purification and biochemical characterization on synthetic substrates of the enzymes from *Flavobacterium johnsoniae* and partially wrote the manuscript.

Paper IV: First author. I planned and performed the bioreactor productions, purifications, enzymatic assays and *Escherichia coli* work. I analyzed the data and wrote the manuscript.

Abbreviations list

AA	auxiliary activities
AOX1	alcohol oxidase 1
CAZy	carbohydrate-active enzyme database
CAZyme	carbohydrate-active enzyme
CBM	carbohydrate-binding module
CE	carbohydrate esterase
CE1	carbohydrate esterase family 1
CLEA	cross-linked enzyme aggregate
CLEC	cross-linked enzyme crystal
DNA	deoxyribonucleic acid
FA-Ara	5- <i>O-trans</i> -feruloyl-L-arabinofuranosyl
FAE	feruloyl esterase
GH	glycoside hydrolase
GT	glycosyl transferase
LPMO	lytic polysaccharide monooxygenase
MCA	methyl caffeate
MFA	methyl ferulate
MpCA	methyl <i>p</i> -coumarate
MPS	mesoporous silica
MSA	methyl sinapate
P _{AOX1}	AOX1 promoter
pI	isoelectric point
PL	polysaccharide lyase
PTM	post-translational modification
PUL	polysaccharide utilization locus
PULDB	polysaccharide utilization loci database
SBA	Santa Barbara Amorphous
SBA-15	Santa Barbara Amorphous type 15

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1. Introduction

Plant biomass is an abundant and renewable carbon source, and is used as a nutrient source by several microorganisms. The main components in this type of biomass, also called lignocellulose, are typically cellulose (40-50%), hemicelluloses (25-30%), and lignin (15-20%) [1]. Cellulose is a homopolymer of glucose which adopts a crystalline configuration, hemicelluloses are heteropolymers comprised of different types of monosaccharides (carbohydrate building blocks), both pentoses and hexoses, and are often branched and/or appended with various chemical groups such as acetyl and feruloyl moieties. Lignin consists of phenylpropanoid units which form through radical coupling to generate a complex hydrophobic network that both rigidifies the cell wall and protects against pathogens [2]. All three polymers are intertwined and chemically bonded together, making lignocellulose a highly complex and recalcitrant material to degrade.

Due to the complexity of plant biomass and the diversity of monomers and chemical bonds present in its structure, microorganisms able to grow on lignocellulosic materials need to possess arrays of degradative enzymes. Such enzymes, as well as the ones responsible for creating the complex carbohydrates of the plant cell wall, have been classified in the carbohydrate-active enzymes database (CAZy) [3]. Non-catalytic carbohydrate-binding modules (CBMs) are also found in CAZy, as they are important for targeting the enzymes to their substrates. More details on the CAZy database can be found in **Chapter 2**.

Enzymes are biological macromolecules produced by all living organisms, and the ability of enzymes to catalyze chemical reactions are crucial to maintain life as we know it. Through evolution, life has spread over the planet with organisms being found in diverse environments spanning the globe. The diversity of existing environments have led organisms to adapt to highly variable environmental conditions. In microorganisms, the main mechanisms of adaptation are mutations and gene transfer [4]. Microorganisms have evolved to thrive in diverse environments varying in temperature, pressure, pH, and salinity (**Table 1**) [5]. To allow this, enzymes have changed with species evolution to gain activity and stability under the specific conditions in which various organisms live.

Table 1 Growth conditions of different types of microorganisms

	Low	Medium	High
Temperature	psychrophiles $T_{\text{opt}} \leq 15^{\circ}\text{C}$	mesophiles $T_{\text{opt}} \approx 37^{\circ}\text{C}$	thermophiles $T_{\text{opt}} \geq 50^{\circ}\text{C}$
Pressure			barophiles above 100 MPa
pH	acidophiles $\text{pH}_{\text{opt}} \leq 2$	neutrophiles $\text{pH}_{\text{opt}} \approx 7$	alkalophiles $\text{pH}_{\text{opt}} \geq 9$
Salt			halophiles from 2.8 up to 6.2 mol/kg NaCl

T_{opt} : optimal growth temperature; pH_{opt} : optimal growth pH.

Enzymes are biological catalysts comprised of amino acids. A catalyst is a substance that increases the rate of a chemical reaction without itself undergoing any permanent chemical change. The catalytic nature of enzymes implies that a single enzyme molecule can catalyze the same reaction several times. Enzymes do not change the thermodynamics of a chemical reaction and the difference in Gibbs free energy (ΔG) between product and substrate must be negative for the reaction to be feasible (**Fig. 1**). Enzymes make chemical reactions faster by lowering the activation energy. Detailed information and description of the underlying mechanisms are beyond the scope of this work but can be found in literature [6,7]. The behavior of many enzymes, including the ones discussed in this thesis, can be described by the Michaelis-Menten equation (**Box 1**) [8].

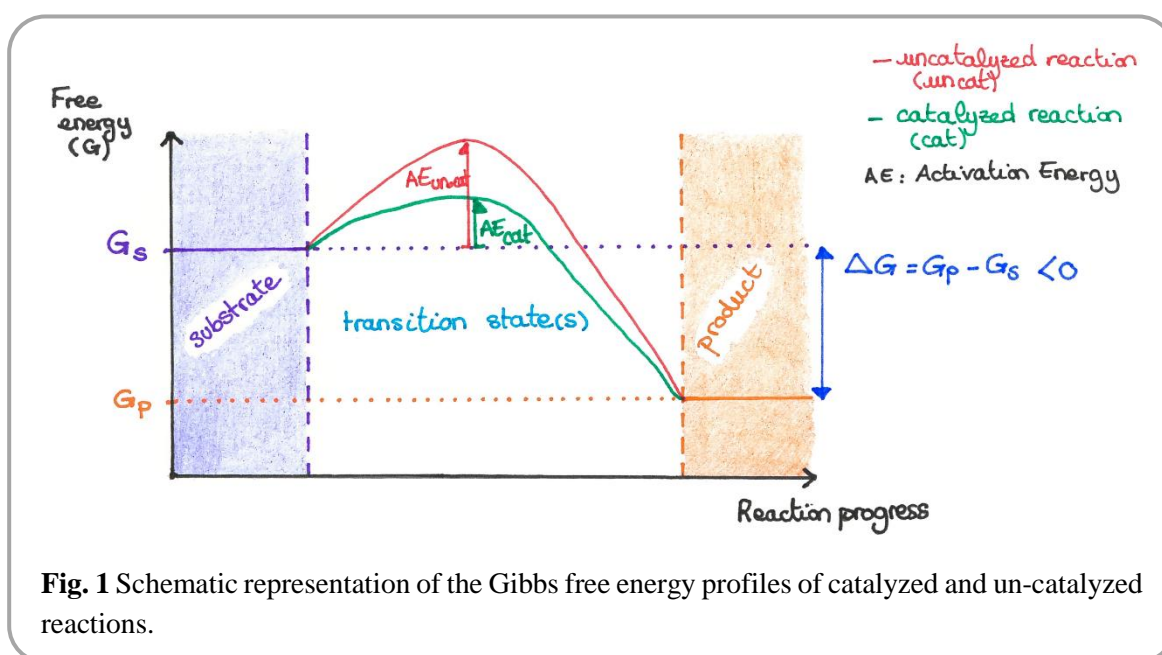


Fig. 1 Schematic representation of the Gibbs free energy profiles of catalyzed and un-catalyzed reactions.

Box 1 Michaelis-Menten kinetic constants and their equations

The Michaelis-Menten equation describes the steady state kinetics of an enzymatic reaction, depending on the initial substrate concentration (Equation (1)). Fitting experimental data to this equation allows for the determination of two constants: K_m and V_{max} . The Michaelis constant K_m is the substrate concentration at which half of the catalytic sites are occupied, and relates to the enzyme's affinity for the substrate. V_{max} is the maximal reaction velocity. Knowing V_{max} and the initial concentration of the enzyme, $[E_0]$, the turnover number, k_{cat} , can be calculated through Equation (2). The ratio k_{cat}/K_m , referred to as the catalytic efficiency, is often used to compare enzymes. In some cases, the experimental data cannot be fitted to Equation (1), due to, for instance, the presence of inhibitors in the reaction. Several inhibition mechanisms, and their corresponding modified equations, exist. Substrate inhibition was the only type of inhibition encountered during this thesis work, and can be described by Equation (3) which allows for the determination of V_{max} , K_m and of the inhibition constant, K_{si} .

$$(1) \quad V = \frac{V_{max} [S]}{K_m + [S]} \quad (2) \quad k_{cat} = \frac{V_{max}}{[E_0]} \quad (3) \quad V = \frac{V_{max} [S]}{K_m + [S] \left(1 + \frac{[S]}{K_{si}}\right)}$$

Enzymes are proteins, as such, they are made of amino acids, and their sequences are encoded by deoxyribonucleic acid (DNA) (**Fig. 2**). In order to yield an active enzyme, the amino-acid chain needs to be properly folded and, in some cases, modified by post-translational modifications (PTMs). Among PTMs, glycosylation has been of interest in this thesis and will be discussed in **Chapter 4**. Proper folding, because it can affect binding capabilities between the enzyme and substrate, is crucial for enzymatic activity and specificity [6].

Nowadays, by combining empirical testing of enzymatic activity with the utilization of powerful computer-based tools, bioinformatics is able to make predictions about protein folding and enzymatic activity based on gene sequence [9]. Annotation of genes with their corresponding putative protein function are becoming more accurate as experimental data is generated and fed back into increasingly sophisticated models (**Fig. 2**). Web-based tools made functional prediction publicly available and although automated annotations are not always accurate and rely on sequence homology with known protein-folds, they remain a powerful tool for guiding novel-enzymes discovery [9–12].

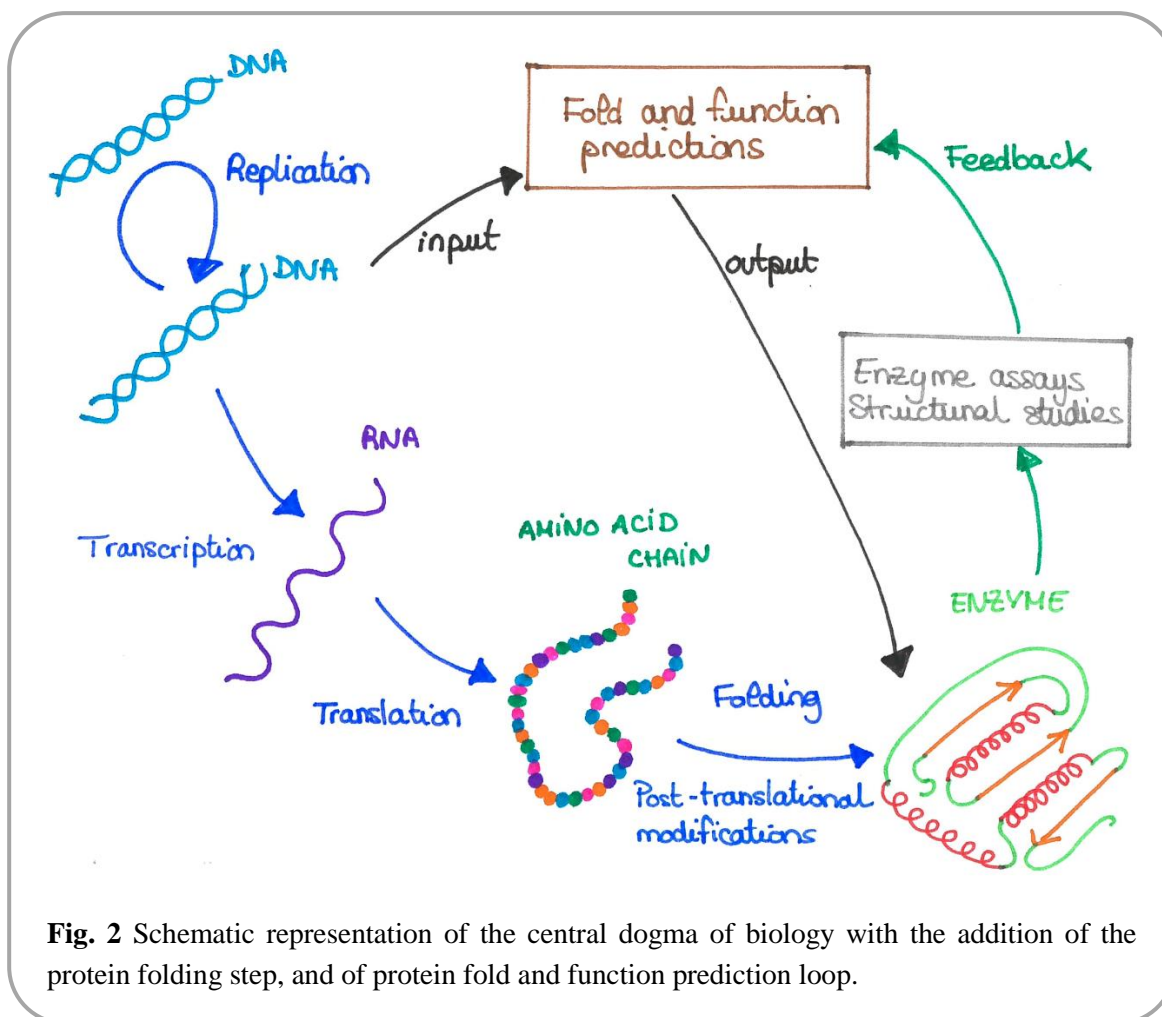


Fig. 2 Schematic representation of the central dogma of biology with the addition of the protein folding step, and of protein fold and function prediction loop.

Enzymes are often highly efficient and specific for one chemical reaction, and their catalytic activity is tightly linked to the protein structure [6]. However, enzymes can display substrate promiscuity, sometimes considered as side or secondary activity. This phenomenon is linked to the mechanisms of evolution, which rely, in part, on random mutagenesis happening during DNA replication. A weak enzymatic activity towards a different substrate or for a different chemical reaction, can indeed facilitate the evolution and development of new enzymes after mutations. Which, in turn, may allow the host-microorganism to use different substrates as feed source, adapt, and thrive in various novel environments [13]. Enzyme promiscuity has even been exploited to generate enzymes capable of catalyzing non-natural reactions, some examples of these novel enzymes have been reviewed recently [14].

In order to determine the activity of a putative enzyme, genetic sequence and structural features can be helpful, but experimental data is needed to confirm the actual catalytic activity. Experimental assessment of enzymatic activity, preferably performed on a purified enzyme, requires that the scientists have access to a suitable substrate and to a way of monitoring the reaction progress. Moreover, especially when testing enzyme activity using synthetic-substrates, one should keep in mind that: (i) the substrate used might not possess all the required structural features for the enzyme to act on it, and (ii) many enzymes have side-activities and the activity tested might not be the main one of the enzyme.

In the context of the current efforts to switch from petrochemistry-based processes to more sustainable ones, enzymes are an asset to help reach this objective. The aims of this thesis work were to study various carbohydrate-active enzymes in order to evaluate how their characteristics (*e.g.* structural, chemical), native or not, can influence their properties and degree of fitness for industrial applications, as well as to evaluate the impact of immobilization on their behavior. These aims were translated into the following three research questions, (i) How do native characteristics of enzymes influence their properties and degree of fitness for industrial applications? (ii) How does the chosen protein production host influences the resulting enzyme properties? (iii) How do different enzymes behavior compare, before and after immobilization?

My thesis work focused on carbohydrate esterases (CEs), and in particular feruloyl esterases (FAEs). Esterases will be presented in **Chapter 2**, starting by the general reaction they catalyze, some of their industrial applications as well as some structural features of these enzymes. The CAZy classification will be briefly presented before focusing on FAEs and their role in biomass degradation. The ability of FAEs to perform synthetic reactions, which was investigated in **Paper I**, will also be presented.

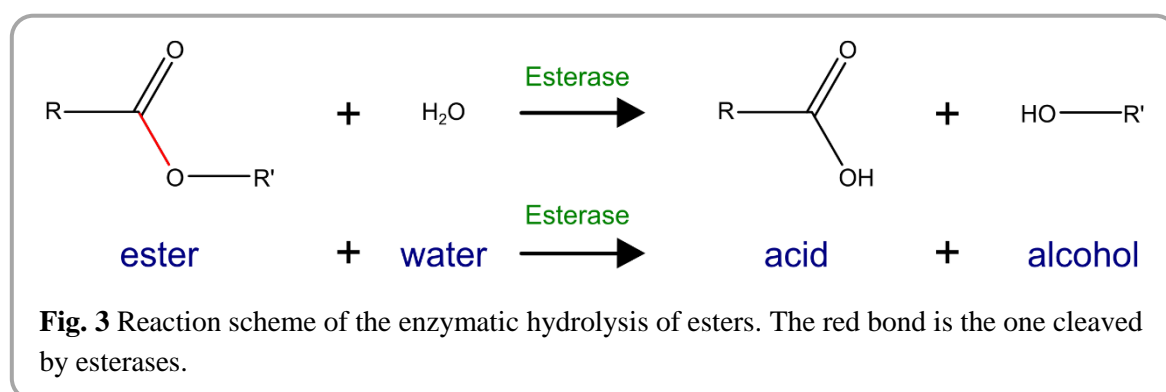
Owing to their outstanding reaction specificity, enzymes are very powerful tools. However, they are also very sensitive to the reaction conditions, and depending on the envisioned industrial process modifying known enzymes or finding novel enzymes might be needed. In **Chapter 3**, ecological niches and strategies to find novel enzymes as well as the importance of functional annotations will be presented. The microbial genomic feature of clustering genes together, which was exploited for **Paper III**, will be introduced. Finally the importance of biochemical characterization of putative enzymes will be emphasized, in relation to the studies presented in **Paper III** and **Paper IV**.

Following experimental confirmation of an enzyme activity, in order to produce it in large quantities, and to render the process more economically feasible, heterologous protein production is often used, and will be presented in **Chapter 4**. Advantages and limitations, as well as some common host microorganisms will be mentioned, focusing on the ones used in **Paper II** and **Paper IV**. The influence on enzyme properties of one post-translational modification, *N*-glycosylation, which was studied in **Paper II**, will then be discussed in relation to the importance of host selection when producing heterologous enzymes.

The last two chapters will focus on enzyme immobilization, which has also been used in order to decrease the costs of industrial processes. **Chapter 5** will introduce the main immobilization techniques and their respective advantages and drawbacks. In **Chapter 6**, the focus will be put on mesoporous silica particles, which were used as the immobilization support in **Paper I** and **Paper II**. Features of this type of immobilization supports, as well as their influence on the immobilization process will be presented. Finally, based on the data from **Paper I** and **Paper II**, the consequences of immobilization in mesoporous silica on enzymatic activity will be discussed.

2. Esterases

Esterases are enzymes with a hydrolytic activity targeting ester bonds, which upon cleavage release a carboxylic acid and an alcohol (**Fig. 3**). Such enzymatic activity is important for lignocellulose degradation as some polymers, in particular hemicelluloses, can carry ester-linked side-groups. Some of the ester-linked groups found in biomass are acetic acid, ferulic acid and glucuronic acid [15]. It has been shown that the presence of these side-groups on plant biomass can both prevent the action of polysaccharide-cleaving enzymes and be involved in covalent linkages with lignin, thereby increasing lignocellulose recalcitrance [16,17]. Some esterases are therefore of great importance to biomass depolymerization as they can remove the side-groups that may otherwise prevent the action of several other enzymes, and aid in separating the polysaccharides from lignin. As a result, these biomass acting enzymes are integral members of the CAZy database (section 2.3) [3], and belong to the carbohydrate esterases class.



2.1 Industrial applications of esterases

A myriad of esterases other than the ones acting on lignocellulose exist. The diversity of their possible substrates have given esterases applications in various industrial sectors including food and feed, pharmaceutical, cosmetic, pulp and paper, bioethanol production (CAZymes), leather production, photography and printing inks, or chemical remediation,

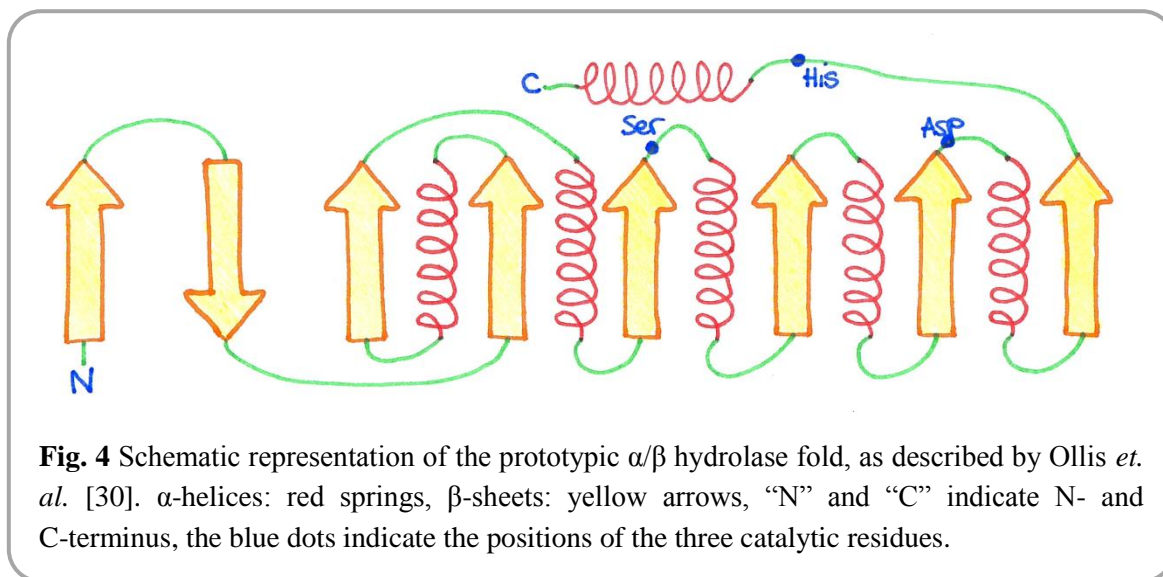
and several reviews have been dedicated to present these industrial applications [18–24]. While in some cases the enzymes are used to get rid of ester-linked compounds hindering further processing, in other cases, the released product is the compound of interest. This can be the case for tannases and FAEs because their reactions liberate phenolic acids, which have been shown to be bioactive compounds and therefore of interest to various industries, as described in the recent review by Heleno *et. al.* [25].

Among esterases, FAEs were of special interest in this work. Their hydrolytic and synthetic activities are presented in sections 2.4 and 2.5, respectively. The main industrial usage of FAEs are found in (i) bioethanol production, where FAEs are important enzymes in xylan degradation [26], (ii) pulp and paper industry, where enzymatic supplementation with FAE improved delignification of pulp and reduced the need for chemical bleaching [27], and (iii) feed industry, where FAEs can be applied to remove ferulic acid, which improves the digestibility of crop residues [28]. In addition, some hydroxycinnamic acids have been shown to possess anti-oxidant activities [29], and could be of interest to the cosmetics industry. However, in order to improve and facilitate formulation, it might be necessary to modify their properties (*e.g.* hydrophilicity), which can be achieved enzymatically by taking advantage of the synthetic abilities of FAEs [21].

2.2 The α/β hydrolase fold

As mentioned in the introduction, the amino acid sequence and the fold proteins adopt are linked. However, similar tertiary structures have been obtained despite low sequence similarity, as for instance with the α/β hydrolase fold [30]. The α/β hydrolase fold, which has been defined a clan in the Pfam database (<http://pfam.xfam.org/>; [31]; AB_hydrolase; CL0028) is wide-spread among enzymes of different activities, including FAE, tannase, acetyl esterase, acetyl xylan esterase and lipase activities [31]. Catalytic domains adopting an α/β hydrolase fold typically display 5-11 β -strands organized according to the prototypic architecture presented in **Fig. 4**, and their catalytic activity is supported by three amino acids composing the catalytic triad [30,32]. Interestingly, the residues forming the catalytic triad in α/β hydrolases are found in very similar topological and three-dimensional positions [30]. In agreement with their similar positions in the folded structure, the three catalytic residues are found in the same order in the amino-acid sequence: first the nucleophile, then the acid, and finally the base [30]. While the base, histidine, is conserved in the triad [30], the other two residues, the nucleophile (Ser, Cys, Asp) and the acid, can be different depending on the enzyme [33]. Another feature of the α/β hydrolase fold, typical of serine hydrolases, is the nucleophilic elbow, defined by a consensus sequence, **G-X-S-X-G** around the nucleophile (where **G** is glycine, **X** can be any amino acid and **S** is serine, the nucleophile; also referred to as Sm-X-Nu-X-Sm, where Sm is a small residue, X can be any amino acid and Nu the nucleophile) [30,34]. The nucleophilic elbow consensus sequence leads to the protein forming a sharp hairpin around the nucleophile [30]. Despite conservation of the nucleophilic elbow, enzymes belonging to the α/β hydrolases family display low sequence

identity [32]. The three-dimensional configuration adopted by α/β hydrolases is well-suited for several types of chemical reactions [30], and the sequence variations observed might be linked to the different substrate specificities of these enzymes, leading to the presence of different substrate binding sites. One of the consequences of the low sequence similarity, is that it renders it difficult to assign a putative function to α/β hydrolases based on sequence comparison alone. The fact that this fold is found in several enzymes having diverse activities demonstrate that it is very efficient and could also be an explanation for the presence of side-activities in α/β hydrolases.



2.3 The carbohydrate-active enzymes classification

Carbohydrate-active enzyme are classified in their dedicated database, CAZy (<http://www.cazy.org/>; [3]), and are often referred to as carbohydrate-active enzymes (CAZymes). The CAZy database was made available in 1998, and has been continuously updated since then. This database provides information about various enzymes, sorted in five different classes based on their activities (**Box 2**), as well as about non-catalytic modules which are often found together with CAZymes, the carbohydrate-binding modules. Each class in the CAZy database is divided into several families, and enzymes are assigned to their family based amino acid sequence similarities [3]. Predicted or observed structural information, as well as biochemical characterization of the CAZymes is also linked when available.

Box 2 Enzyme classes in the CAZy database

- Glycoside Hydrolases (GHs): hydrolysis and/or rearrangement of glycosidic bonds
- Glycosyl Transferases (GTs): formation of glycosidic bonds
- Polysaccharide Lyases (PLs): non-hydrolytic cleavage of glycosidic bonds
- Carbohydrate Esterases (CEs): hydrolysis of carbohydrate esters
- Auxiliary Activities (AAs): redox enzymes that act in conjunction with CAZymes

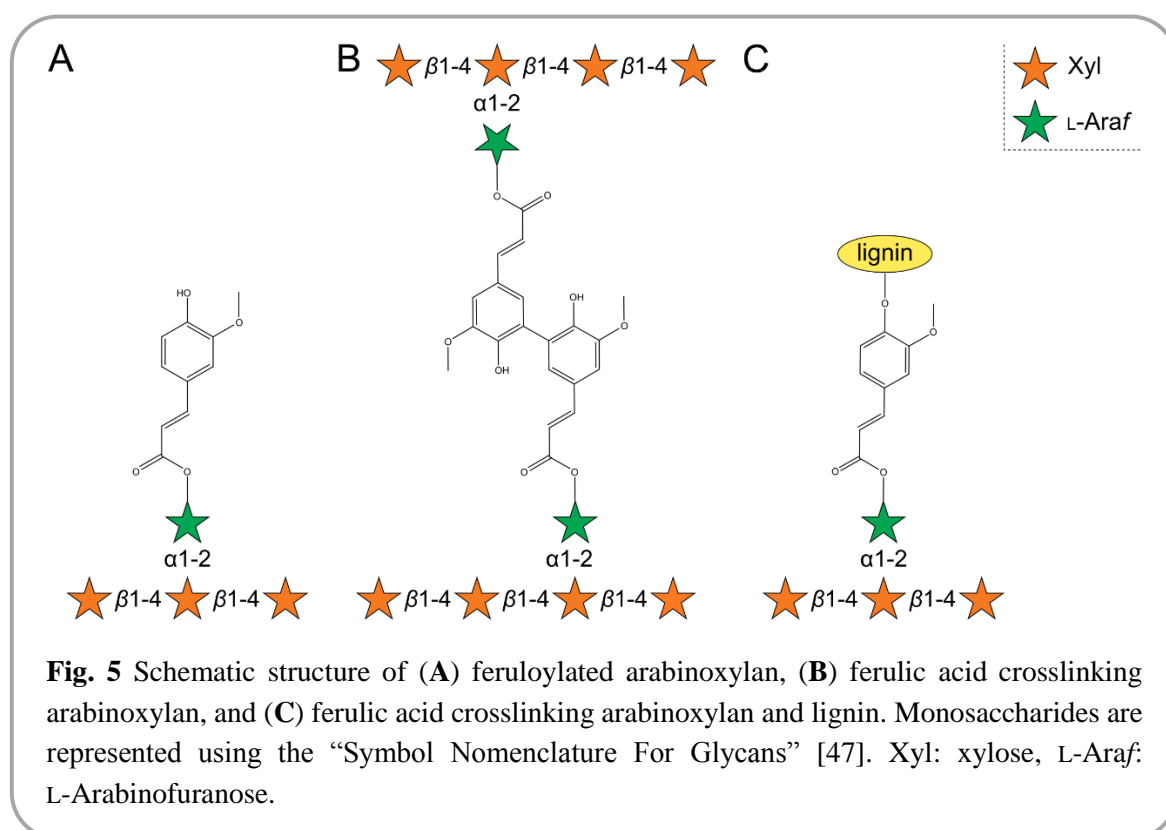
Due to the inherent specificity of enzymes and the complexity of plant biomass, enzyme cocktails to degrade lignocellulose contain an array of CAZymes. Commercially available cellulolytic cocktails were historically based on the collected enzymes secreted by the cellulose degrading fungus *Trichoderma reesei* [35]. Cellulases and other CAZymes produced by microorganisms act in conjunction to degrade biomass and it has even been proven that some CAZymes are able to act synergistically [26,36,37]. In some cases, the synergistic effects observed were induced or enhanced by the presence of physical links between the enzymes, through structures like cellulosomes [38,39]. The advantages of physical proximity between enzymes will be discussed more in detail in **Chapter 3**, and have been investigated in **Paper III**.

The work presented in this thesis focuses on enzymes belonging to the carbohydrate esterase class from the CAZy classification, and especially enzymes belonging to carbohydrate esterase family 1 (CE1). CE1 is currently the CE family containing the largest diversity of enzymatic activity, with at least seven different enzyme commission numbers (EC numbers; acetyl xylan esterase (EC 3.1.1.72); cinnamoyl esterase (EC 3.1.1.-); feruloyl esterase (EC 3.1.1.73); carboxylesterase (EC 3.1.1.1); S-formylglutathione hydrolase (EC 3.1.2.12); diacylglycerol O-acyltransferase (EC 2.3.1.20); trehalose 6-O-mycolyltransferase (EC 2.3.1.122); as of October 7, 2019) [3]. This family also contains all the FAEs currently present in the CAZy database.

2.4 Feruloyl esterases (FAEs)

Feruloyl esterases belong to the α/β hydrolase superfamily and possess the canonical Ser-His-Asp catalytic triad. Despite their similar catalytic activity, FAEs are diverse in protein sequence, with some FAEs being closer in sequence similarity to lipases than to other FAEs, as demonstrated in 2004 by Crepin *et al.* in the first FAE classification attempt [40]. In that classification, other FAEs were shown to be similar to tannases. Sequence similarity between FAEs and tannases is also exemplified by the fact that they are currently listed as a single family “Tannase and feruloyl esterase” in the Pfam database (PF07519) [31]. Since the first classification in 2004 [40], new biochemical characterizations of FAEs have led to the creation of other classification systems [41–43]. These classifications emphasize that feruloyl esterases are very diverse and suggest that FAEs probably evolved from various esterases, such as lipases, tannases or acetyl xylan esterases, and that fungal and bacterial FAEs might have followed different evolution pathways [43,44].

On lignocellulosic substrates, FAEs catalyze the release of ferulic acid, which is found ester-linked to arabinosyl moieties (5-*O-trans*-feruloyl-L-arabinofuranosyl; FA-Ara; **Fig. 5A**) decorating the xylan backbones in commelinid monocots [15]. The FA-Ara groups can further form bonds with other FA-Ara, leading to the formation of di-ferulate structures, and consequently crosslinking of closely positioned xylan polymers (**Fig. 5B**) [45]. It has also been suggested that FA-Ara could participate in crosslinking xylan to lignin through bonds with coniferyl alcohol (**Fig. 5C**), as has been demonstrated *in vitro* [46]. By cleaving the ester linkage between ferulic acid and arabinose, FAEs could help disentangling lignocellulosic biomass. FAEs also facilitate hydrolysis of xylan, and have been shown to act in synergy with xylanases [26,37].



FAEs have been shown to be active on hydroxycinnamic acids, such as methyl ferulate (MFA), methyl caffeate (MCA), methyl sinapate (MSA) and methyl *p*-coumarate (MpCA) (**Fig. 6**). These four acids are commonly used as synthetic substrates to detect FAE activity, and formed the basis of the FAE classification introduced by Crepin et. al. [40].

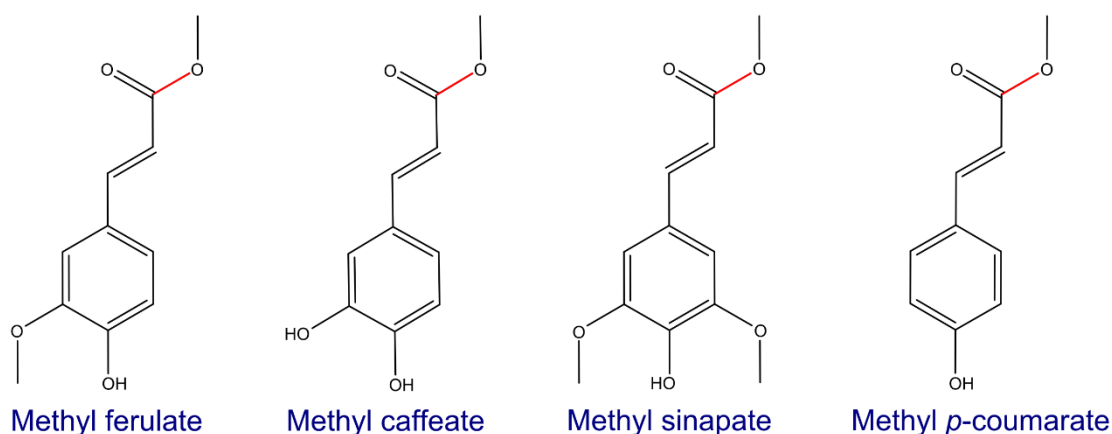
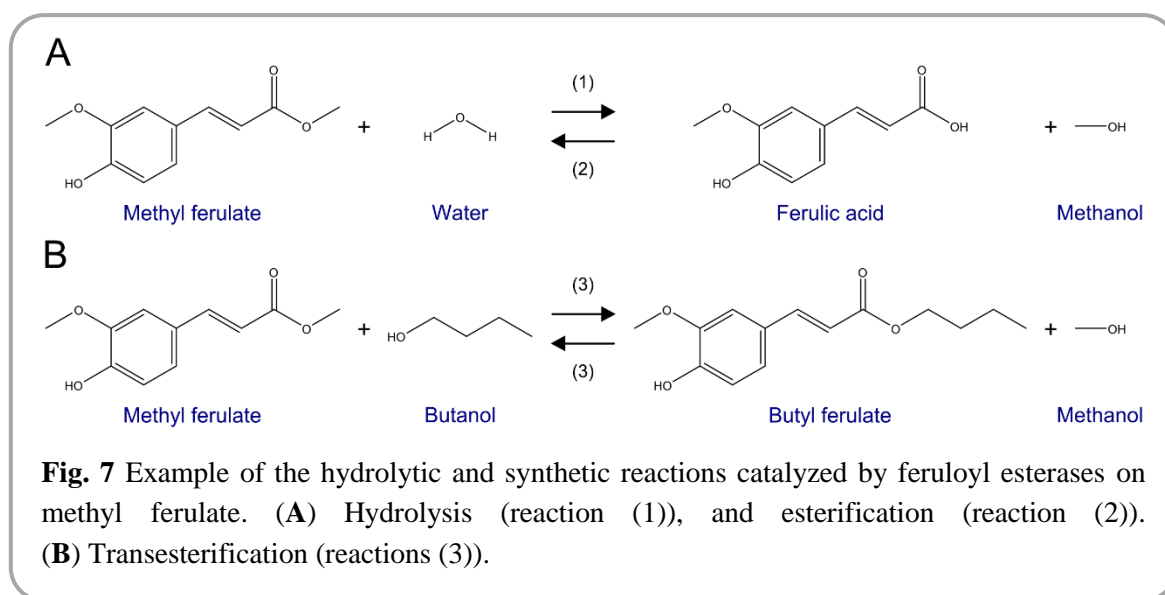


Fig. 6 Chemical structures of four synthetic substrates commonly used to test FAE activity. The red bond is the one cleaved by feruloyl esterases.

2.5 Synthesis using hydrolytic enzymes

Biomass, and its components, have also attracted interest from the chemical industry, where monosaccharides would not be used as feed sources for microorganisms, but as starting compounds for further chemical modifications. In nature, most glycosidic bonds are synthesized by glycosyl transferases (GTs), which form a class in the CAZy database (**Box 2**). Due to some limitations in substrate availability and reaction specificity, not all desired reactions are easily feasible using GTs [48]. Chemical modification of mono- and polysaccharides can be a long and tedious process requiring multiple protection/deprotection steps in order to obtain the desired molecule, yielding processes unsuitable for large-scale production [49,50]. In order to address this, one of the strategies has been to modify glycoside hydrolase (GH) activities to make the enzymes perform their typical reactions in reverse, *i.e.* making them synthesize the bonds they usually break [48,51]. In a similar way, the hydrolytic FAEs have been investigated for their ability to synthesize ester linkages through esterification (**Fig. 7A**), or transesterification (**Fig. 7B**).



Making a hydrolytic enzyme perform synthetic reactions usually relies on altered thermodynamic equilibria, using co-solvents to replace water, in order to favor the synthetic reaction. Co-solvent use can be combined with other approaches such as enzyme engineering (favoring synthesis/removing hydrolysis), or immobilization. The thermodynamic aspects of FAE and lipase synthesis reactions on hydroxycinnamic acids have been thoroughly reviewed [52]. Several fungal and bacterial FAEs have been tested for their synthetic abilities, using different solvent systems such as micro-emulsions with or without detergents, alcohol solutions or ionic liquids [52,53]. One of the main challenges faced when trying to use enzyme in non-water based reaction systems is enzyme stability in such solvents [54,55], as indeed the vast majority of enzymes have evolved to be active in aqueous environments. The transesterification capabilities of a commercially available FAE (E-FAERU, from Megazyme) were investigated in **Paper I**, and will be presented and discussed in **Chapter 6**, together with enzyme immobilization.

3. Enzyme discovery

As mentioned in the introduction, because of the variety of process conditions encountered industrially, an array of enzymes possessing the same activity, but functioning in different conditions, is needed. Enzyme engineering can be used to improve or modify the properties of enzymes using methodologies such as directed evolution [56]. However, if the desired catalytic activity exists naturally, nature and microorganisms have already optimized countless enzymes for various reaction conditions. Therefore, in order to find novel enzymes with diverse properties, mining the existing microbial biodiversity, guided by genomic annotations, is a powerful strategy. Two of the studies presented in this thesis relied on this approach, in **Paper III** bacterial multi-domain enzymes annotated as CE6-CE1 were studied and in **Paper IV** putative FAE/Tannase targets of fungal origin were investigated.

3.1 Where to find novel enzymes?

One can find microorganisms in diverse environments such as in the soil, in the digestive tracts of animals, on plants, as well as on decaying plant biomass. Microorganisms have been found growing at very different temperatures (**Table 1**), and some were for instance isolated from tropical soils or Antarctic lakes [57,58]. In order to survive in such conditions, microorganisms need to possess a functional metabolism, and hence enzymes active at various temperatures. In industry, high and low temperatures are relevant as both may prevent contamination by mesophilic microorganisms [5,59]. In addition, at high temperatures ($> 60^{\circ}\text{C}$), the solubility of many chemical compounds is increased and the viscosity of medium is decreased [5]; while at low temperatures ($< 20^{\circ}\text{C}$) the energy requirement for heating are reduced, and heat-sensitive products are preserved [59]. CAZymes originating from thermophiles as well as psychrophiles have been characterized and studied [60,61].

In **Paper II**, the FAE used as a model enzyme to evaluate the impact of glycosylation originated from a thermophilic fungus, *Myceliophthora thermophila* (other names: *Chrysosporium lucknowense*, *Thermothelomyces thermophila* [62]). In **Paper IV**, two fungi

from the *Aspergillus* genus, which genomes had been sequenced and annotated [63], were selected. The two selected fungi were both isolated from soil, with *Aspergillus zonatus* isolated from tropical soil whereas *A. glaucus* was found in the soil of arctic regions.

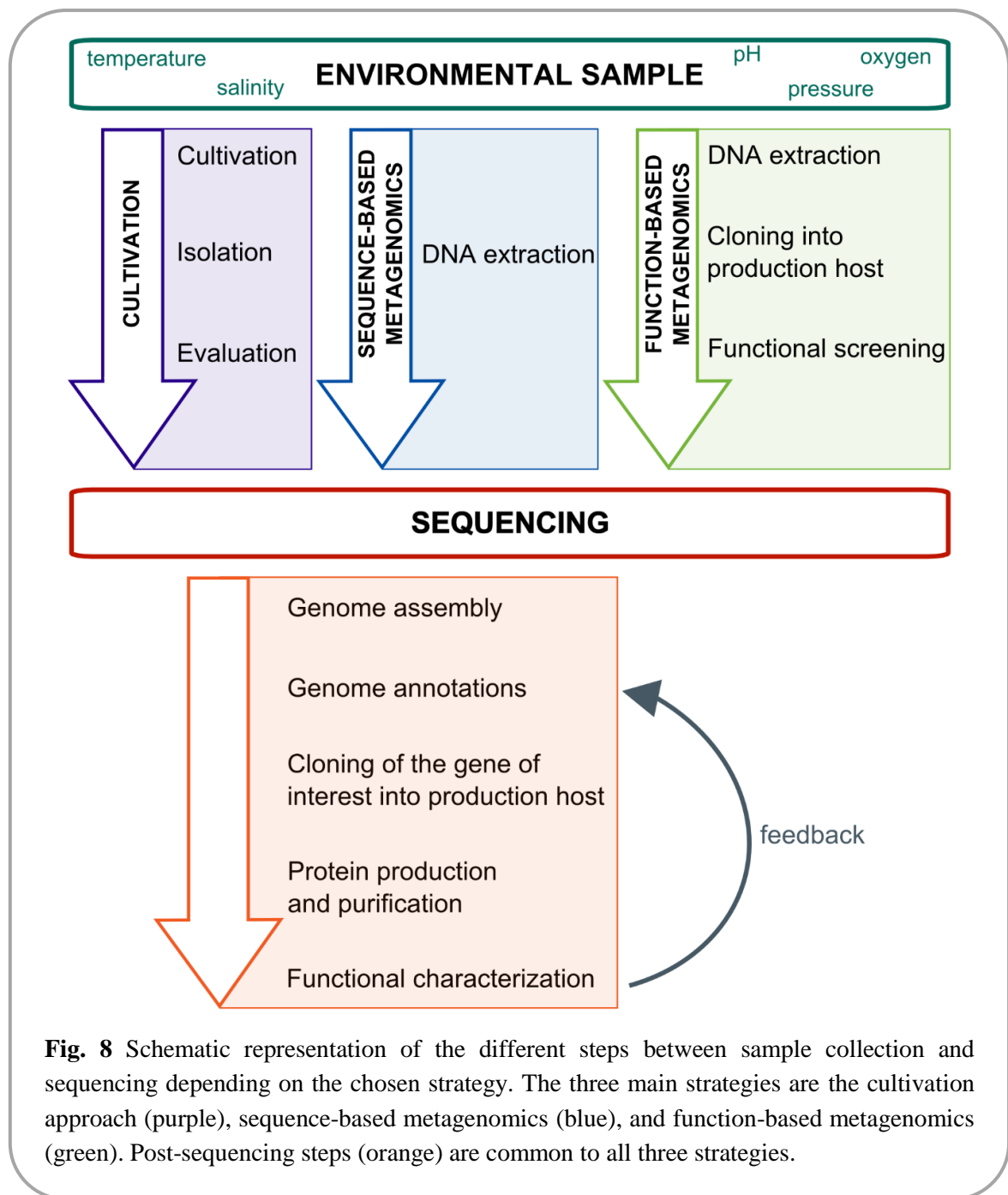
Bioprospecting in soil or on decaying biomass is often performed when the aim is to find novel CAZymes. Another great source of CAZyme-producing microorganisms is the digestive tract of animals, including humans. Most higher organisms do not possess the required enzymes to digest the various carbohydrates found in biomass, but rely on their symbiotic microbiota to utilize it [64]. Among the most studied digestive tract microbiotas are the human-gut microbiota [65] (including extensive studies related to health as reviewed recently [66]), the rumen microbiota of ruminant mammals [67], and the gut microbiota of termites [68]. Among the bacteria present in rumen, human and termite guts, the ones belonging to the Firmicutes and Bacteroidetes phyla are generally dominating [65,67,68]. Enzymes found in human-gut or rumen are likely to be adapted to act best around 37°C. In **Paper I**, the commercial enzyme used to study transesterification and immobilization was coming from a rumen microorganism. In **Paper III**, the investigated enzymes were from *Bacteroides ovatus* and *Flavobacterium johnsoniae*, which both belong to the Bacteroidetes phylum.

3.2 How to find novel enzymes?

Following bioprospecting and sample collection, two major approaches can be followed: microorganism isolation and cultivation, or total DNA extraction for metagenomics analysis. Both strategies have advantages and drawbacks.

Isolation and cultivation of microorganisms allows for selection of strains that for example are able to grow at defined temperature, pH, and utilize a specific substrate as a nutrient source. Using this strategy, one can also evaluate and select the best growing microorganism in the selected conditions, and/or further select the microorganism producing the most protein or having the highest enzymatic activity (**Fig. 8**). Such studies have been conducted by our research division in collaboration with a research group from the Food Industrial Research Institute, Hanoi, Vietnam, and led to the identification of several novel fungal strains which proved to be efficient in hydrolyzing rice straw [69], as well as being able to perform hydrolysis at high temperatures (some enzymes retained up to 90% of their activities after 20 min at 70°C) or in acidic conditions (pH 3.0) [70]. After identification of promising lignocellulose degraders, the genome(s) of the selected target(s) can then be sequenced. Because only one or a few microorganisms at a time are being sequenced, the amount of genomic data to treat is smaller and subsequently easier to assemble and annotate than if total sample DNA was sequenced. Genomic data can sometimes be combined with transcriptomic and/or proteomic data, providing even more insight on the biomass degrading strategies of the microorganism. For instance, this was performed on a thermophilic fungus, *Malbranchea cinnamomea*, which is able to grow on various types of plant biomass [71].

Combined genomic and transcriptomic data gave insight on the strategies employed by this fungus to degrade different biomass sources, and may allow identification of industrially relevant thermostable enzymes. In summary, the cultivation and isolation approach has the advantage of reducing the size of genomic data generated, and also allows to directly focus on species which ability to grow on, and therefore degrade, biomass has been experimentally observed. However, the major drawback of this approach is that it only allows identification and investigation of the species that we manage to grow in lab conditions.



Metagenomics on the contrary allows for gaining information on non-cultivable microorganisms. Investigating microbes that we cannot readily grow in standard lab

conditions can be crucial since in a recent review Pham and Kim reported on approximately 99% of soil bacteria to currently be non-cultivable [72]. Metagenomics is a method based on the extraction and sequence determination of all of the DNA contained from an environmental sample, regardless of whether the microorganisms it contains are cultivable in lab conditions or not. Two strategies can then be applied, sequence-based or function-based (**Fig. 8**). Sequence-based metagenomics (sometimes referred to as random metagenome sequencing [73]), make use of the recent progresses of sequencing technologies, sequence-reads assembling tools, and automatic annotation servers in order to handle the big datasets generated by sequencing the total DNA content of the sample.

Function-based metagenomics incorporate an activity screening step in the workflow before the sequencing step (**Fig. 8**). For this, DNA fragments are cloned into an expression host, generating a library of cells containing diverse genetic elements. The abilities of the microorganism colonies are then evaluated, and clones exhibiting the desired features can subsequently be sequenced. Sequencing only the genetic elements which conferred the targeted function (*e.g.* a specific enzymatic activity), drastically reduces the amount of DNA to be sequenced and can guide genome annotation. However, this strategy relies on the availability of a suitable protein production host, activity-screen, and high-throughput screening platforms. For more technical details on metagenomics and their usefulness in enzyme discovery, the reader is referred to the recent review by Ufarté *et. al.* [73].

No matter the approach chosen in order to find novel enzymes from environmental samples, following gene identification and annotation, functional evaluation of the corresponding protein has to be performed. The target gene has to be cloned into a suitable host, the protein to be produced and purified before its activity can be evaluated. The importance of this final step is discussed in section 3.4.

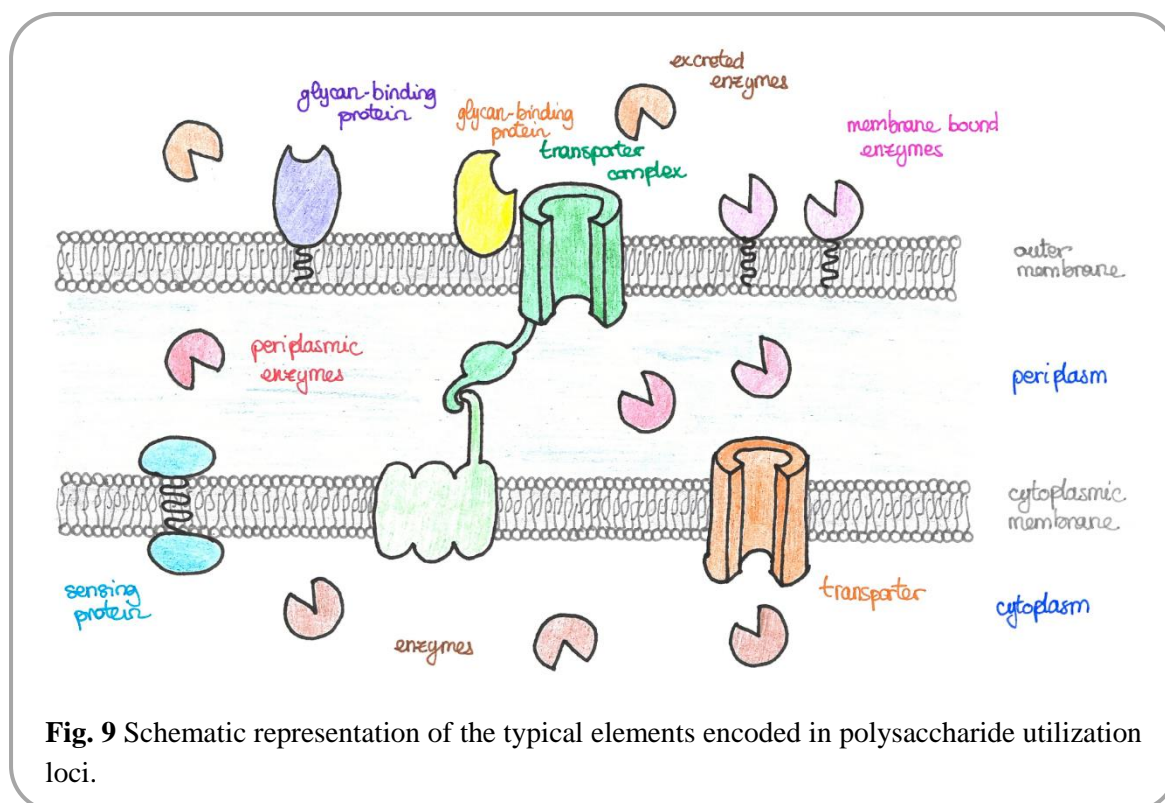
3.3 Hints from genome organizations

Some features in the genome organization can help in identifying genes as putative enzymes. Indeed, it has been shown that several CAZymes are multi-domain proteins, consisting of a catalytic domain and one or more other modules. Glycoside hydrolases, in particular, are often associated with one or more carbohydrate-binding module [71,74,75]. CBMs are thought to help enzymatic degradation of polysaccharides through three major modes of action: favoring and prolonging physical proximity of enzyme and substrate, specifically binding to the polysaccharide on which the catalytic module is active, and disrupting the substrate crystallinity through non-catalytic mechanisms [76]. The structural features underlying the action of CBMs, as well as their potential for biotechnology applications have been reviewed [76,77]. Owing to the function of CBMs, binding to a polysaccharide polymer, it is common that one of the catalytic domain(s) they are linked to possesses an activity on that polymer.

A subclass of multi-domain proteins is multi-catalytic enzymes, *i.e.* proteins which possess two (or more) distinct catalytic domains. The advantages of such architecture have different explanations depending on the nature of the fused enzymes. Substrate channeling effect has been proposed as a reason for the increased activities observed when the product of one of the catalytic module is the substrate of another module [78], as has been demonstrated with two enzymes of the ribulose monophosphate pathway that were fused *in vitro* [79]. Artificially fused constructs have also been created using CAZymes, and the resulting proteins were shown to act synergistically [80,81]. Multi-catalytic CAZymes have also been found in microorganisms and were shown to be highly efficient enzymes. The multi-catalytic CelA from *Caldicellulosiruptor bescii* – composed of (from N- to C-terminal) one GH9 endoglucanase domain, three CBMs, and one GH48 exoglucanase domain – was shown to outperform a mixture of endo- and exoglucanases used in commercial cocktails in the degradation of a model cellulose substrate (Avicel) [82]. The performance for chitin hydrolysis of the multi-catalytic chitinase, ChiA (from *F. johnsoniae*, comprising two GH18 chitinase domain) exceeded the action of the corresponding two domains [83]. Indeed, the full length ChiA hydrolyzed over 80% of β -chitin and over 20% of α -chitin in 24h, compared to 30% and 5% on β - and α -chitin, respectively, using the corresponding mixture of the N- and C-terminal domains [83]. In **Paper III**, we studied two of such proteins, predicted to possess an N-terminal CE6 domain, and a C-terminal CE1 domain. The biological role of the enzymes was tested by monitoring the hydrolysis of corn cob by a xylanase when adding the CE domains, separately or together, or the full length enzyme. The addition of the full length enzyme from *B. ovatus* resulted in the release of 30% more reducing sugar equivalents compared to the addition of the corresponding amount of an equimolar mix of the two domains. Although using the same methodology, the enzyme from *F. johnsoniae* did not yield the same results, the data from *B. ovatus* demonstrates that there are situations where producing multi-catalytic enzymes could be beneficial for the bacterium. Experiments did not allow the determination of the underlying mechanism to this activity increase, but the hypothesis was that it could be related to coordinated or synergistic activity of the domains, possibly thanks to physical proximity.

The enzyme targets from *B. ovatus* and *F. johnsoniae* were both located within gene clusters dedicated to polysaccharide degradation (**Paper III**). Such clusters are referred to as polysaccharide utilization loci (PULs), and are another genomic feature that can help in curating automated functional annotations. In bacterial taxa, clusters of genes encoding the proteins of a pathway, or needed to ensure a specific function (*e.g.* degrading a polysaccharide), are a common genomic organization [84]. In the case of PULs, the clustered genes encode proteins of complementary functions aiming at degrading a specific type of polysaccharide. The proteins encoded in PULs typically consist of glycan-binding and sensing proteins, degrading enzymes, and transporters allowing the internalization of specific carbohydrates (**Fig. 9**) [85]. Recently, PULs targeting diverse glycans such as xyloglucan, yeast mannan, cellulose, chitin, and xylan have been characterized [83,86–89]. Focusing on the two bacteria studied in **Paper III**, the polysaccharide utilization loci

database (PULDB, <http://www.cazy.org/PULDB/>, [90]) lists more than 30 and 100 PULs for *F. johnsoniae* UW101 and *B. ovatus* ATCC8483 (new assembly), respectively (as of September 5, 2019). The two multi-catalytic enzymes studied in **Paper III** were located in PULs from *B. ovatus* and *F. johnsoniae* which are believed to target xylan and have been described in literature [89,91]. Neither of the enzymes had been previously biochemically characterized. Therefore these two putative multi-catalytic esterases were investigated, focusing on their potential biological and biochemical roles.



3.4 Limitations of predictions: the importance of experimental evidence

Protein sequence, fold, and function are tightly linked, however, proteins with low sequence similarity can adopt the same fold, and proteins with the same fold can have different enzymatic activities [30]. In addition, some automatic annotation tools do not allow to fully discriminate between two or more enzymatic activities. This is in particular the case of FAEs, which can be found in the Pfam database under the “Tannase and feruloyl esterase” family, PF07519 [31], and in the CAZy database under CE1, which contains other enzymes than FAEs [3]. In the case of FAEs, more specific functional annotations might not be possible yet due to the relatively low number of biochemically characterized enzymes, as well as the low number of available protein structures (only 13 characterized enzymes and 3 protein structures were reported in the CAZy database, as of September 5, 2019; although not all published FAEs studies were included in CAZy yet). Biochemical characterization is hence always required in order to confirm the activity of an enzyme. In **Paper IV**, all five selected targets were annotated as putative Tannase/FAE. Although full biochemical characterization

was not performed on all enzymes, qualitative activity screening revealed that two of the targets were tannases, two were FAEs, while the fifth one appeared to be an acetyl esterase. These results exemplified the fact that annotations are not yet always able to discriminate between FAEs and tannases. However, the enzyme presented as an acetyl esterase in **Paper IV**, might actually possess FAE or tannase activity on native biomass although this activity was not detected on synthetic substrates. In **Paper III**, we were not able to confirm, nor disprove that the CE1 domain of the *B. ovatus* enzyme (*BoCE1*) was an FAE. Indeed, if an enzyme is able to cleave a synthetic substrate, one can conclude it is active, but the opposite is not true. In the specific case of *BoCE1*, we were only able to demonstrate that this domain has an esterase activity using 4-nitrophenyl-acetate and 4-methyl-umbelliferyl-acetate, on which many esterases are active, as observed in **Paper IV**. In addition, despite low catalytic efficiencies on synthetic substrates, supplementation with *BoCE1* had a significant boosting effect on the hydrolysis of corn cob by a xylanase. We can, however, not exclude that this domain could be active on ester-linked ferulic acid as found in plant biomass since natural (**Fig. 5**) and synthetic substrates (**Fig. 6**) could bind differently to the enzyme.

Another important fact to keep in mind is that the putative functions assigned to proteins are predicted with models relying on similarities with known enzymes or proteins. Hence, functional annotations are based on experimental data and cannot predict hitherto unknown activities or folds. One recent example of this phenomenon is the case of lytic polysaccharide monooxygenases (LPMOs), which were initially annotated in the CAZy database as GH61 enzymes or non-catalytic CBM33 proteins [92]. Following the discovery by Vaaje-Kolstad *et.al.* that CBM33 proteins could oxidatively degrade crystalline chitin [93], a similar observation followed for the GH61 enzymes on crystalline cellulose [94]. These two families were subsequently reclassified as “auxiliary activity” (AA; families 9 and 10, respectively) [92].

4. Heterologous protein production

When the enzyme of interest is encoded by a gene originating from a non-cultivable microorganism, heterologous protein production is used (*i.e.* production in a different microorganism than the one the protein is originating from). But even if the native-organism of the target enzyme is cultivable, producing the enzyme in its native-host might not be the most effective strategy. Therefore, it is common that heterologous protein production is used at both lab and industrial scales. In this thesis work (**Papers II-IV**), three microorganisms were used for enzyme production, *Escherichia coli*, *Pichia pastoris* and *Myceliophthora thermophila*, and will be the focus of this chapter.

4.1 Advantages and limitations of heterologous production

Heterologous protein production was rendered possible by the development of DNA manipulation technology in the late 1960s (the early developments were summarized in 1974 by Clark [95]). Since then, this technique has been constantly developed, owing to its numerous advantages over homologous protein production. Indeed, when homologous production might involve growing animals, plants or pathogenic microorganisms, using genetic engineering one can – in principle – produce any protein in a suitable microbial host.

Heterologous protein production, because it often uses a well-known microorganism as a host, is generally easier, cheaper, and allows to reach higher production yields than homologous production. Microorganisms that are used for heterologous production are often suited for large-scale cultivation, and “tool boxes” allowing for molecular biology work have been developed for them (**Box 3**). Such tool boxes are commercially available for *E.coli* and *P. pastoris*, and one has recently been developed for *M. thermophila* [96]. These tool boxes, and their corresponding methodologies, allow for the development of production strains which can, for instance, be inducible (*e.g.* the *E.coli* “(λDE3)” strains which allow the induction of the production of proteins controlled by the T7 promoter [97]), be protease-deficient (*e.g.* *P. pastoris* SMD1168H [98], *M. thermophila* C1 [96]), or have low background levels of protein production and excretion (*e.g.* *M. thermophila* C1 [96]). All

these features enable reaching high protein production yields and, in turn, facilitate downstream processes such as protein purification. When genetic tool boxes, methodologies and strains are available, it allows for cloning of the gene, followed by production tests of the target protein in a timely manner. In addition, if one uses well-known hosts, industrial cultivation conditions suitable for the microorganisms are often known. All this work participates in making heterologous protein production cheaper and easier than homologous production.

Box 3 Elements of genetic tool boxes

- Selection markers (*e.g.* autotrophy- or antibiotic-based)
- Promoters (identification of strong/weak promoters)
- Vectors (development of high-transformation-rate vectors)
- Gene disruption tools (*e.g.* knock-out methods)

When producing a protein in a heterologous host, two main types of limitations exist. These limitations relate to codon utilization, and post-translational modifications and can hamper protein production and/or the function of the resulting proteins. Amino acid sequences are encoded in DNA by triplets of nucleotides forming codons, and this genetic code is nearly universal [99,100]. However, it is redundant, meaning that one amino acid can be encoded by more than one codon. Studies have shown that the frequency of codon usage is non-random and varies depending on species [101]. When codon usage differs too much between two microorganisms, or if one uses a non-canonical genetic code [100], heterologous protein production might be impossible. In the past decades, the cost of synthetic genes has greatly decreased, rendering the optimization of DNA sequences based on the codon usage affordable, and often leading to drastic improvements in protein production yields [102]. Recent studies have, however, shown that codon optimization strategies aiming at utilizing only most common codons might be counterproductive for co-translational protein folding [103–106]. Therefore, selecting a host with a similar codon utilization to that of the native organism benefit abundant production of functional protein. Some heterologous genes have also been shown to be toxic to the host cells, and strategies to counteract this issue in *E. coli* (*e.g.* periplasmic production) have been reviewed [107,108]. Protein folding problems, leading to protease degradation and/or protein aggregation, may be a consequence of lack of proper post-translational modifications. Indeed, in order to adopt their active folded structure, some proteins require chemical modifications, such as disulfide bonds, and side-chains addition. Some common PTMs, as well as their biological roles have been reviewed by Mann and Jensen [109]. Among PTMs, glycosylation was of particular interest in the present work. The effect of *N*-glycosylation on the characteristics of an FAE was studied in **Paper II**, and its importance will be presented in more details in section 4.3. Advantages and drawbacks of using heterologous protein production are specific to the chosen host. Some hosts, including the ones used in **Papers II-IV**, are presented in section 4.2.

4.2 Hosts for heterologous production

Various systems can be used for heterologous production of proteins, and some hosts might be better suited than others for the production of a specific protein. Hosts can be microorganisms (such as bacteria, yeast, or filamentous fungi) or more complex organisms (*e.g.* insect cells, mammalian cells, transgenic plants or transgenic animals) [110]. The host-organism is chosen depending on the target protein properties: molecular weight, amino-acid sequence, and need for post-translational modifications for instance. This section is mainly focused on the three microorganisms used in **Papers II-IV**: *E. coli*, *P. pastoris* and *M. thermophila* (**Fig. 10**). Information about some other systems can be found in the review by Demain and Vaishnav [110].

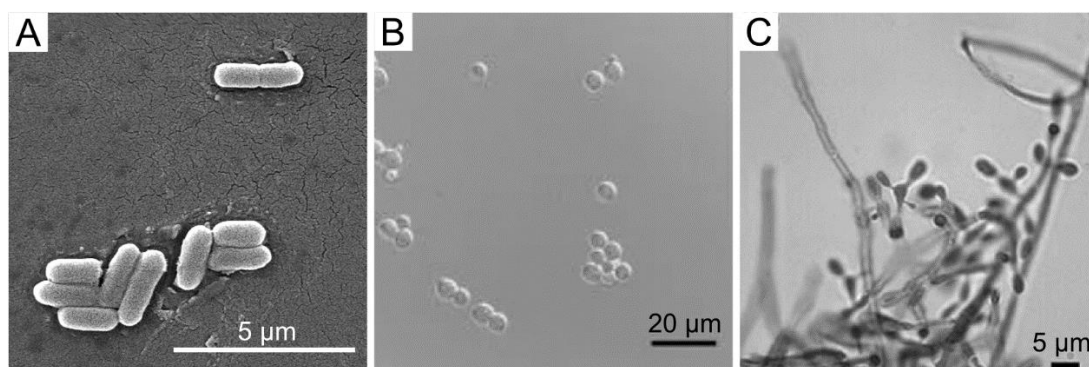


Fig. 10 Microscopy images of the three microorganisms used for protein production. (A) *Escherichia coli* cells. (B) *Pichia pastoris* cells [111]. (C) *Myceliophthora thermophila* cells [112].

Among bacterial hosts, *E. coli* is by far the most used and studied. *E. coli* has a rapid growth, is easy to cultivate, allows for rapid protein production at high yields, and its genetics are well understood [110]. Several strains and plasmids have been developed in order to expand the range of proteins that can be heterologously produced by *E. coli* [108,113]. In addition, methods to work with genes or proteins which are toxic to this bacterium [107], or with proteins that are produced as inclusion bodies [114], methods to increase protein solubility and final yields through co-production of chaperones [115], as well as genetic engineering enabling *E. coli* to glycosylate proteins [116,117], have been described and extensively reviewed. The development of these techniques, together with strain engineering, allowed to counteract many of the drawbacks of *E. coli* as a production host. However, at high specific growth rates, *E. coli* is known to produce acetate, which can be inhibitory to the cells [110]. Another drawback of this bacterium is that, as many other Gram-negative bacteria, its membrane contains endotoxins [118], which have long been known for their bioactivity [119], and might complicate downstream processing of the protein depending on its intended application. Other bacterial hosts, such as Gram-positive bacteria from the *Bacillus* genus, have been investigated. *Bacillus* species possess advantages: they do not produce endotoxins, they have protein excretion machineries, and several species have obtained the

“generally recognized as safe” status from the US Food and Drugs Administration [110,120]. Until now, the low production yields usually reached when producing heterologous proteins have limited the industrial use of *Bacillus* species, but several strain engineering strategies aiming at increasing production yields have been used and/or are under development [120]. *Bacillus* species are therefore considered as a promising bacterial host for future applications.

Compared to bacteria, yeast cells offer advantages such as efficient excretion of proteins, incorporation of disulfide bonds, and natural ability to introduce glycan-chains on the proteins. Among yeasts used for heterologous protein production, two species are dominating: *Saccharomyces cerevisiae* and *P. pastoris* [110]. Because of its historic utilization in baking and brewing processes, *S. cerevisiae* has been extensively studied and is now used as a model eukaryotic microorganism, with several existing models of its metabolism [121]. The interest in *P. pastoris*, a methylotrophic yeast, developed more recently due to its great performance for heterologous protein production. Reviews describing existing strains, plasmids, promoters, signal sequences and the glycosylation pathway of this yeast have been published [98,122]. The progress made in metabolic engineering of *P. pastoris* for metabolite production as well as for heterologous protein production has been summarized recently [123]. Compared to *S. cerevisiae*, *P. pastoris* possesses several advantages for heterologous protein production. *P. pastoris* generally produces higher protein yields than *S. cerevisiae*, as exemplified by the therapeutic protein hirudin [124]. As a methylotrophic yeast, *P. pastoris* is able to grow on methanol as the sole carbon source, mostly through its oxidation by the alcohol oxidase 1 (AOX1) [125]. Since this enzyme has a poor affinity for oxygen, when grown on methanol, *P. pastoris* strongly upregulates the AOX1 promoter (P_{AOX1}) [126,127], which in turn allows for tight control of induction and strong production levels of the P_{AOX1} -controlled genes. Finally, although both *S. cerevisiae* and *P. pastoris* are known to produce hyper-mannosylated proteins, on average, the amount of mannose residues introduced by *P. pastoris* is smaller than what has been observed for *S. cerevisiae* [98,128]. This might be an important aspect and a desired feature for heterologous protein production. Indeed, in **Paper II**, I demonstrated that the homologously produced enzyme – which carried shorter glycan-chains than the version heterologously produced in *P. pastoris* – had a better activity and thermostability (see section 4.4).

A third type of microorganisms used as hosts for heterologous protein production are filamentous fungi. These microorganisms are also able to introduce glycan-chains on proteins and to excrete them [129]. Among them, *Aspergillus* species as well as *Trichoderma reesei*, are most used [110,130–132]. Recently, new fungal hosts have been investigated, such as *M. thermophila*. A toolbox for molecular biology manipulations has been developed for this host [133], and strains with low protein excretion background have been developed, making *M. thermophila* an interesting alternative to other fungal hosts [96]. Although filamentous fungi have mainly be used for the production of fungal proteins, partly because of their abundant production of proteases [131], progress in strain engineering

should enable the development of fungal strains suited for heterologous production of proteins from non-fungal sources.

4.3 Influence of glycosylation on proteins and enzymes

Glycosylation is one of the most known and studied PTM (which can also happen co-translationally), during which glycan-chains are attached to the amino acid chain. The most common types of glycosylation are *N*-glycosylation and *O*-glycosylation [117]. In *N*-glycosylation, the glycan-chain is bound to an asparagine residue (**Fig. 11**), while in *O*-glycosylation, glycan-chains are linked to the oxygen atom in serine or threonine residues, usually in proline-rich regions. *N*-glycosylation is more common than *O*-glycosylation [117], and consensus sequences of potential glycosylation sites are well defined (Asn-X-Ser/Thr; where X is not a proline), whereas no consensus sequence have been identified for *O*-glycosylation sites. The synthesis pathway of glycan-chains has been reviewed [98,128,134].

In the case of *N*-glycosylation, the first steps, happening in the endoplasmic reticulum, are common to plants, yeast and mammal cells. These first steps involve the transfer of a pre-assembled $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ (**Fig. 11A**) by an oligosaccharide transferase on the amide group of an asparagine residue of a nascent protein. Glucosidases then remove the three glucose units and one mannose unit is removed by a mannosidase, leaving $\text{Man}_8\text{GlcNAc}_2$ on the protein, a structure known as the core *N*-linked oligosaccharide (**Fig. 11B**). The protein is then transferred to the Golgi, where the synthesis pathways differ between organisms and species [128]. In yeasts, the core oligosaccharide is elongated by several mannosyltransferases, while in mammals and plants various glycosidases and glycosyltransferases are involved in the glycan-chains modification. Glycan-chains termination in yeast is not well understood and several factors (*e.g.* culture conditions, media composition) have been shown to influence it [135–137]. In addition, glycosylation is a heterogeneous process that result in the formation of a population of glycan-chain lengths, as was observed in **Paper II**, where glycan lengths varied depending on the glycosylation site as well as the production organism (**Table 2**).

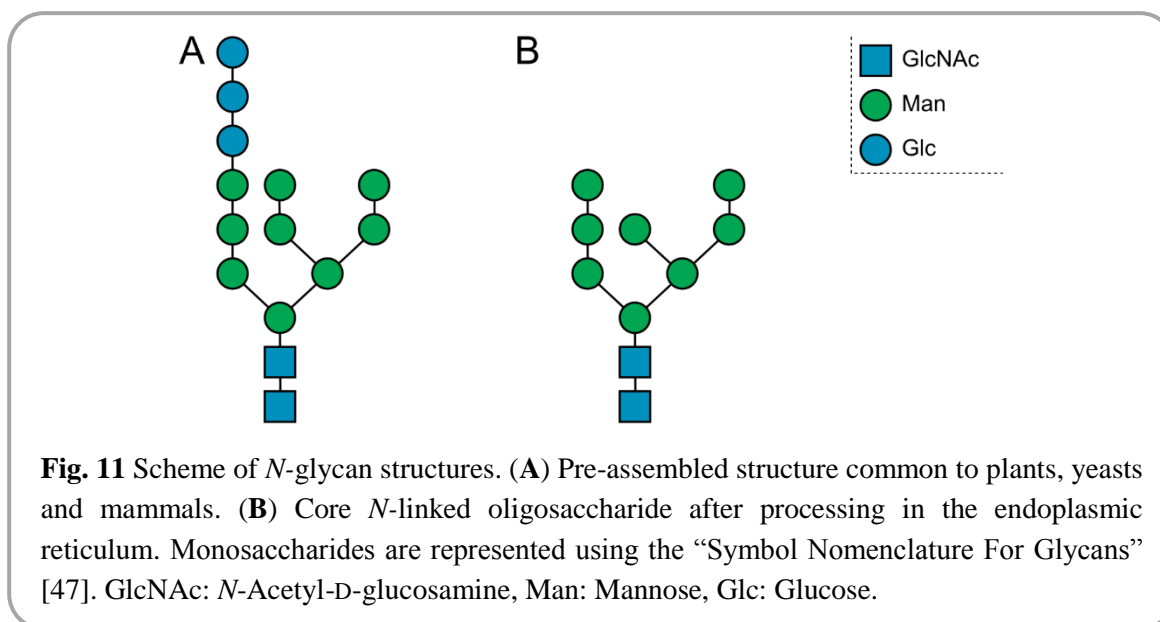


Table 2 Glycan-chain lengths distribution obtained by mass spectrometry analysis in **Paper II**

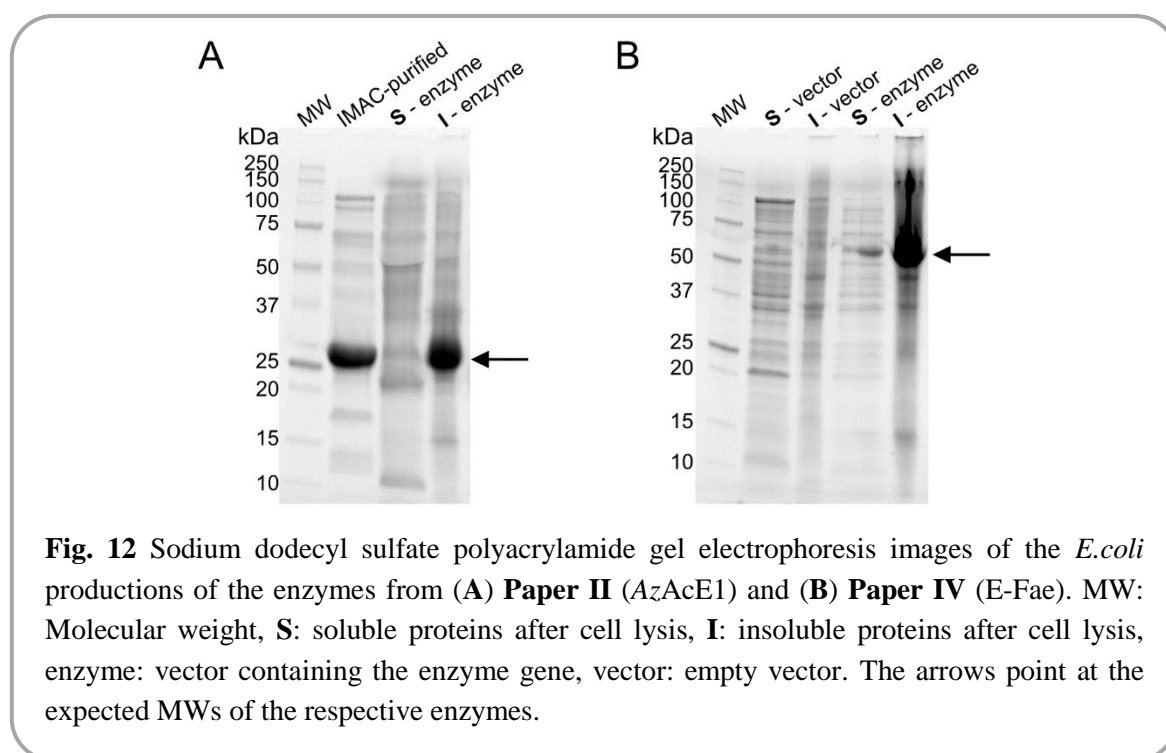
Production organism	Asn 117 glycosylation site	Asn 179 glycosylation site
<i>M. thermophila</i>	HexNAc ₂ Hex ₃₋₉	HexNAc ₂ Hex ₈₋₁₀
<i>P. pastoris</i>	HexNAc ₂ Hex ₉₋₂₁	HexNAc ₂ Hex ₈₋₁₂

The presented glycan structure ranges accounted for more than 90% of the relative structure distributions observed at the corresponding glycosylation site. Hex: hexose. The original data can be found in **Paper II** (Additional file 1. Fig. S1 and S2).

Glycosylation is a key concept in the pharmaceutical industry as glycosylated proteins account for nearly 70% of all approved protein-based drugs [128]. Indeed, *N*-glycosylation appears to have the greatest impact on the efficacy of therapeutic proteins in terms of immunogenicity, anti-inflammatory role, receptor binding, and pharmacokinetics [128,138–141]. Thus, considerable efforts have been made to understand the effects of *N*-glycosylation on proteins, as well as the synthesis pathways and the composition of glycan-chains. This has enabled the development of yeast strains (*S. cerevisiae* and *P. pastoris*) able to produce recombinant proteins with human-like glycan patterns [142], and prokaryotic strains able to perform *N*-glycosylation are being developed (*E. coli*) [116,117,143]. In addition to its role in medical applications, glycosylation has been shown to affect folding [144–148], stability [149], aggregation [150,151], substrate binding [152], structural dynamics [153], and catalytic activity of proteins and enzymes [154]. These results have increased the awareness on the importance of glycosylation for proteins function. However, most research studies have focused on either the presence or lack of glycan-chains, and little is known about the influence of glycan-chains composition and length. In **Paper II** I demonstrated that the glycan-chains composition do have an impact on the properties of *MtFae1a*, an FAE from *M. thermophila* (as discussed in section 4.4).

4.4 The importance of protein production host selection

Selecting a suboptimal production host for a protein can result in low production yields, or no production of functional protein. In **Paper II** and **Paper IV** proteins of fungal origin were produced in *E. coli*. Despite co-production with chaperones, most of both target enzymes were found as insoluble aggregates after cell lysis (**Fig. 12**). Chaperones (*e.g.* GroEL and GroES, which were used in the discussed studies) are proteins whose role is to assist and facilitate other proteins' folding through various mechanisms [155]. The fact that both enzymes had a tendency to form aggregates might be linked to their lack of glycosylation when produced in *E. coli*. Indeed, as discussed in section 4.2, *E. coli* is natively unable to introduce glycan-chains on proteins, and glycan presence has been linked to a decreased aggregation tendency [150,151]. These studies exemplify how the chosen host affects the protein production yields.



The study presented in **Paper II** stemmed from work in the EU-project OPTIBIOCAT, where differences were reported for an FAE when produced in two microorganisms, leading to the hypothesis that those differences arose from variations in the glycosylation patterns of the enzymes. In **Paper II**, I studied three versions of the feruloyl esterase *MtFae1a*, obtained by homologous protein production in *M. thermophila*, and heterologous protein production in *P. pastoris* and *E. coli*. The *E. coli* produced enzyme was not glycosylated and had the poorest activity and stability of all three versions. This was expected since glycosylation is known to be important for thermostability, and several enzymes, including FAEs, were less stable or less active when lacking glycan-chains [147,156]. The other two *MtFae1a* versions were glycosylated, and mass spectrometry analysis of glycan-chains revealed significant length differences between the two proteins (**Table 2** and **Paper II**). Interestingly,

significant differences were also observed between these two enzyme versions, especially in terms of optimal temperature for activity, and thermal unfolding midpoint where the FAE produced in *M. thermophila* outperformed the one produced in *P. pastoris* by 10°C and 8°C, respectively. To the best of my knowledge, this was the first time that thermostability differences due to differences in glycan-chain compositions alone were shown for an FAE. Hence, I concluded that in order to produce a glycosylated protein, selecting any host that is able to glycosylate proteins, such as *P. pastoris* in **Paper II**, might not be sufficient to obtain a protein with native properties. This can be of utmost importance when thermostability is a desired feature of the enzyme.

Heterologous protein production has been key to bringing several protein drugs and enzymes to the market by increasing protein production yields, and therefore, reducing costs. Nowadays, owing to increased rates of microorganisms' discovery, and development of engineered production strains, choosing the most suitable production host for a particular protein should become a standard step in setting-up heterologous protein production.

5. Enzyme immobilization

Enzyme immobilization has been defined as: “the process in which a soluble enzyme is confined into space to generate an insoluble, reusable enzymatic species” [157]. Immobilization of enzymes to be used in industrial processes is often required in order to allow their recovery and reuse. Although the immobilization of enzymes adds development costs to the process it usually leads to costs reductions when the whole process is considered (**Table 3**). Cost reductions are due to increased stability of the enzymes, simplified downstream processes, catalyst reusability, and also to the possibility to use immobilized enzymes in continuous processes [158].

Table 3 Comparison of different aspects of industrial processes depending on the use of free or immobilized enzymes

Industrial process properties	Impact of using immobilized enzymes (compared to free enzymes)
Development costs	Higher
Mass transfer	Similar or decreased*
Enzyme stability	Might increase*
Downstream processing	Easier and cheaper
Enzymes reuse	Possible, usually simple and efficient
Continuous process	Possible
Overall costs	Usually lower

*depending on the immobilization technique used

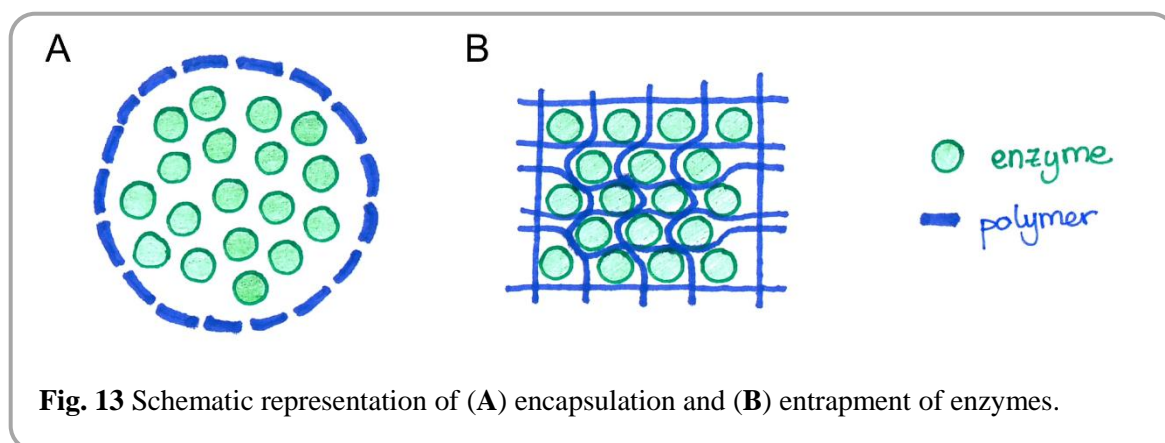
Immobilization can also affect, positively or negatively, enzymes properties such as catalytic activity, overall stability, substrate selectivity, reaction specificity, and inhibitor sensitivity. Several techniques have been developed for immobilizing enzymes and these rely on four main modes of interactions: (i) encapsulation and entrapment, (ii) enzyme cross-linking, (iii) covalent binding, and (iv) physical adsorption. The immobilization techniques will be presented in this chapter, and their respective advantages and disadvantages are summarized in **Table 4** [159–162].

Table 4 Summary of some advantages and disadvantages of the four major immobilization methods

Immobilization method	Advantages	Disadvantages
Encapsulation and entrapment	Shield enzymes from the bulk Enzyme structure not affected	Diffusion limitations Enzymes might leak
Cross-linking	Strong binding Can increase enzyme stability Support-free	Might alter enzyme structure Use of toxic reagents Diffusion limitations
Covalent binding	Strong binding Can increase enzyme stability Various support material / functional groups	Might alter enzyme structure Can be complex and costly
Physical adsorption	Inexpensive and simple Enzyme structure not affected	Enzymes might leak Non-specific interactions

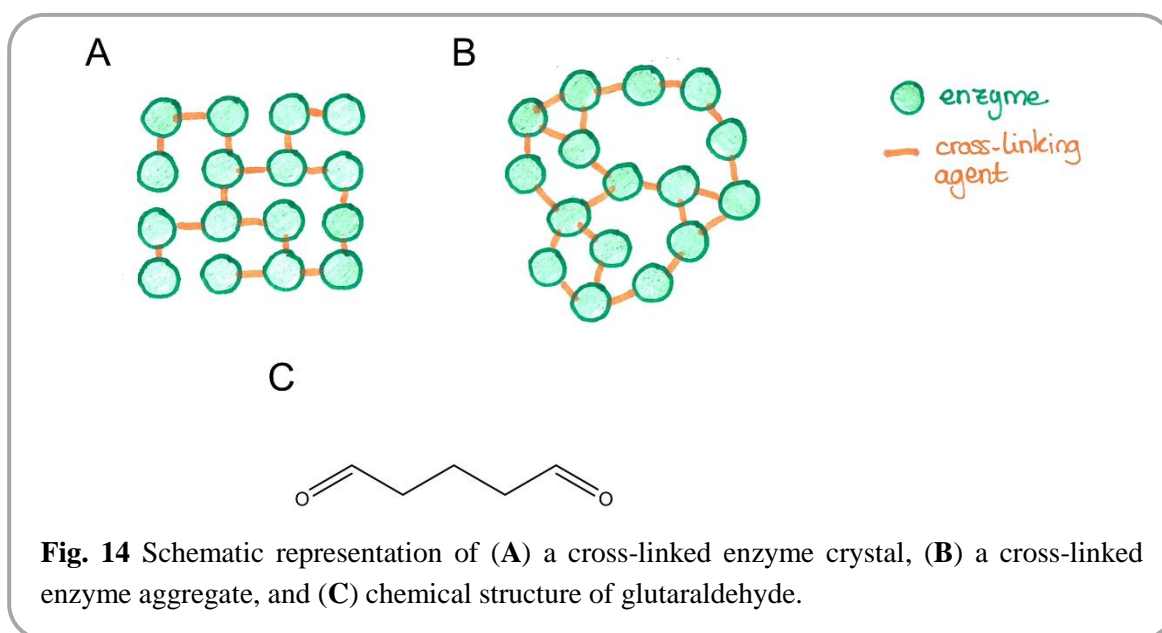
5.1 Encapsulation and entrapment

Encapsulation and entrapment refer to methods where the enzymes are physically confined by membranes (**Fig. 13A**) or within a polymer matrix (**Fig. 13B**). There is no clear definition differentiating these two terms and they have sometimes been used interchangeably [163,164]. The term entrapment has also been used to refer to the immobilization of enzymes inside the pores of a solid support [165]. In my thesis, however, encapsulation and entrapment will not be used in the latter case. Entrapment will only refer to immobilization techniques where enzymes are present during the polymerization step when preparing the matrix. The polymers used for encapsulation and entrapment should form permeable structures, allowing substrate and product diffusion, while retaining the enzymes. Natural polymers such as alginate, agar, chitosan and carrageenan have often been used for immobilizing enzymes [164,166,167]. Some of these natural polymers offer the additional benefits of being cheap, abundant, biodegradable, or bio-compatible [163,167]. Encapsulation and entrapment do not require chemical bonds to the enzyme, therefore the structure of enzymes is usually preserved. The polymer present around the enzymes can also protect them from harmful compounds present in the bulk solution, thereby decreasing enzyme inhibition or other deleterious effects, but can also cause diffusion limitations (*e.g.* of substrate to enzyme, of product from enzyme).



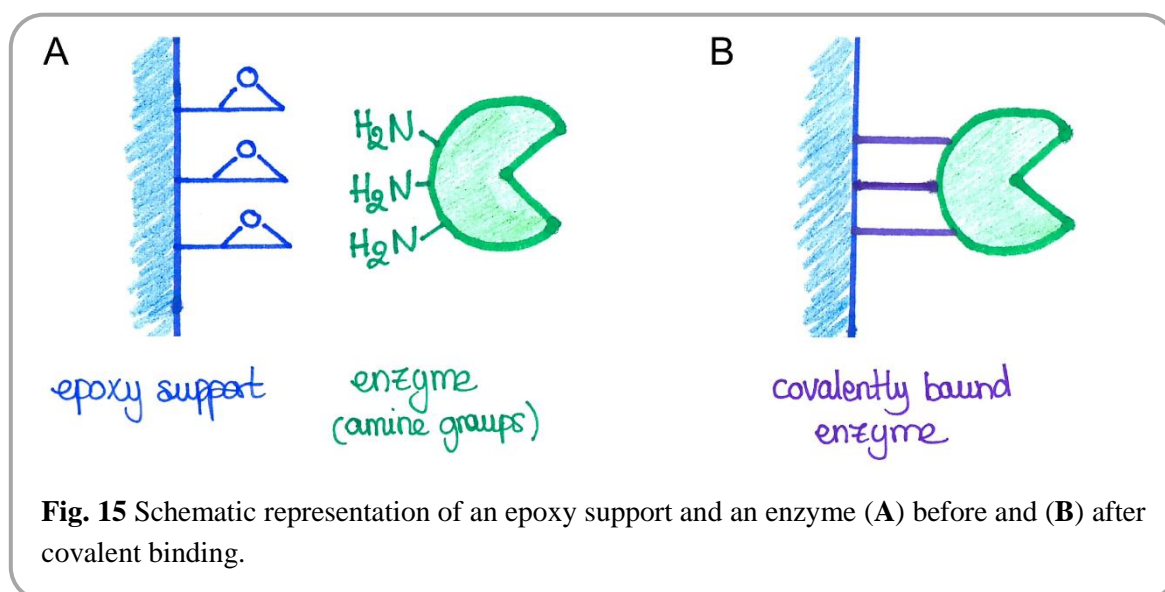
5.2 Enzyme cross-linking

Enzyme cross-linking can be performed on enzyme crystals or enzyme aggregates yielding cross-linked enzyme crystals (CLECs) (**Fig. 14A**) or cross-linked enzyme aggregates (CLEAs) (**Fig. 14B**), respectively. CLEAs have been more commonly used since it is usually easier to obtain enzyme aggregates than crystals. CLEAs also allow different enzymes to be cross-linked together whereas crystallization requires high purity of the target protein. Cross-linking of enzymes is achieved using bi-functional cross-linking reagents such as glutaraldehyde (**Fig. 14C**) or aldehyde dextran [168]. Cross-linking based immobilization techniques have the advantage of being support-free, but might cause alterations to the structure of enzymes because of the harsh conditions during cross-linking [169]. The cross-linking agent glutaraldehyde has also been shown to be able to form intra-molecular bonds, which could either lead to enzyme stabilization, or reduce the flexibility of the enzyme structure too much and be deleterious to its activity [168]. In addition, depending on the enzyme network created through cross-linking, diffusion limitation can also happen [160].



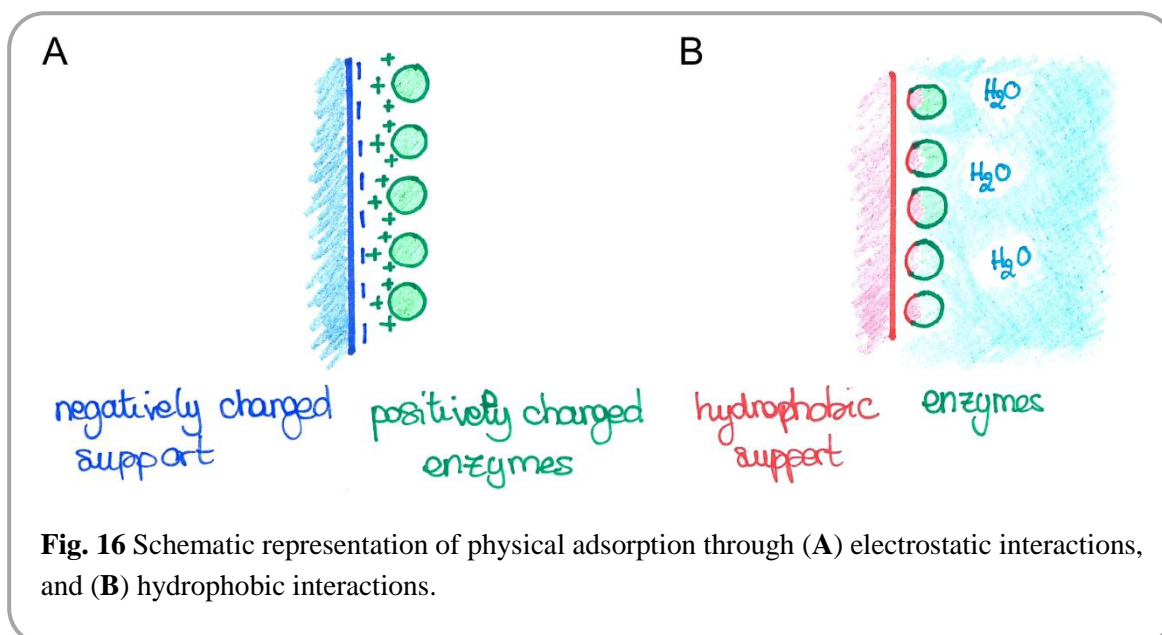
5.3 Covalent binding

In order to immobilize enzymes on solid supports, covalent binding techniques, which involve the creation of a covalent bond between surface residues of the enzyme and functional groups on the solid support material, can be used. Typically, lysine residues on the protein located at the surface of the folded enzymes are targeted to create covalent bonds [170]. On the support side, epoxide groups have been used for commercialized supports (*e.g.* Eupergit, Sepabeads) because of their capacity to bind the amino groups of lysine under mild conditions [171,172] (**Fig. 15**). In addition, epoxide groups can be further modified with other functional groups, their density at the surface of the support can be controlled and varied, and the length of the spacer-arm between the support and the enzyme can also be varied [172–174]. Major advantages of immobilization through covalent binding include the variety of existing supports and functionalization, the strength and stability of the bonds, the improvement of the stability of enzymes through decreased flexibility of their structures.



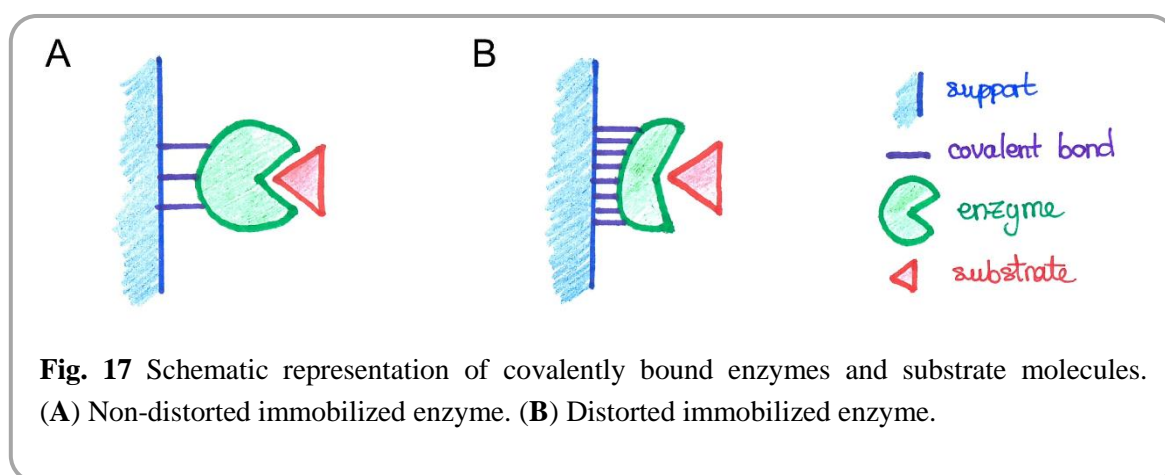
5.4 Physical adsorption

On solid supports, enzymes can also be immobilized by physical adsorption. This method does not involve covalent bond formation between the enzyme and support. Instead, adsorption of enzymes on a solid support depends on the creation of multiple non-covalent interactions between the protein and the support, and is influenced by their intrinsic properties but also by the surrounding environment (*e.g.* presence of solvents, water, ions) [175]. Physical adsorption relies on electrostatic interactions (**Fig. 16A**), hydrogen bonds, and hydrophobic interactions (**Fig. 16B**), which are all weak and reversible interactions. As a comparison, the energy of a disulfide bond (a covalent bond) is 320 kJ.mol^{-1} , while the energy of the interactions involved in physical adsorption are ranging from 4 to 90 kJ.mol^{-1} [175]. The major drawback of immobilization by adsorption is therefore that the enzyme can leak, although large discrepancies between adsorption and desorption mechanism can render the adsorption apparently irreversible [176]. Physical adsorption has been used to immobilize enzymes such as lipases before their utilization in reactions in organic solvents [170]. The development of porous supports, which can confine enzymes and multiply interaction points compared to non-porous materials, has also favored immobilization of enzymes by adsorption. In **Paper I** and **Paper II**, porous supports were used to immobilize FAEs by physical adsorption. In the study presented in **Paper I**, the immobilized enzymes were used in a reaction system consisting mainly of 1-butanol (92.5%; the remaining 7.5% were aqueous buffer), while in **Paper II** the reactions were performed in an aqueous buffer.



5.5 Effects of immobilization on enzymes

Immobilization can affect the biochemical and structural properties of the enzymes [173]. The beneficial effects of immobilization on the thermostability of enzymes is well known [177,178]. However, the underlying mechanisms of stabilization are multiple, and the opposite effect (*i.e.* decreased stability of an enzyme after immobilization) can also be observed. Stabilization of enzymes upon immobilization is often attributed to the “freezing” of its structure through the creation of multiple covalent bonds between the enzyme and the support [179]. However, structure freezing can also cause a loss of enzyme activity if conformational changes are required for catalysis, or if the enzyme structure is distorted when bound to the support (**Fig. 17**) [180,181]. Operational stabilization (*i.e.* under the industrial process reaction conditions) of enzymes has also been reported for enzymes immobilized in porous materials, this will be presented and discussed in section 6.2.



Immobilization has been shown to stabilize multimeric enzymes which can be inactivated during reactions due to dissociation into monomers [182]. Stabilization of multimeric enzymes has been achieved by covalent immobilization [183,184], sometimes coupled with cross-linking strategies [185–187]. Stabilization of the active conformation of enzymes, mainly lipases, by immobilization has been reported recently [188,189].

Immobilization can also modify an enzyme selectivity and/or specificity [190–193]. These changes could be due to either minor distortion of the active site area or to changes in the micro-environment surrounding the immobilized enzymes [173]. Minor changes in the enzyme structure and changes in the surrounding environment have also been proposed to be responsible for the decrease of inhibition (by substrate, product or organic solvents) observed on some immobilized enzymes [194,195].

Enzyme immobilization can be achieved on various supports such as biopolymers, synthetic polymers, mineral materials, which can have different compositions (*e.g.* carbon, magnetic materials, silica), as well as different sizes and morphologies (*e.g.* nanoparticles, nanotubes, graphene or porous materials) [162,196]. The effects of immobilization on enzymes are dependent on the properties of the support (*e.g.* hydrophilicity, morphology), the nature of

the enzyme (*e.g.* amphiphilic, hydrophilic), as well as on the type of interactions existing between the enzyme and the support (*e.g.* covalent, electrostatic). Depending on the most desired property of the chosen enzyme for an industrial process, the immobilization support and technique will therefore vary.

6. Enzyme immobilization in mesoporous silica (MPS)

Each enzyme immobilization support, depending on its composition, size and structure will possess different properties. Important general features of the support materials, and aspects to consider for industrial applications are presented in **Box 4** [197]. Silica, because it possesses most of the features mentioned in **Box 4**, has attracted interest and has been used for the preparation of porous materials.

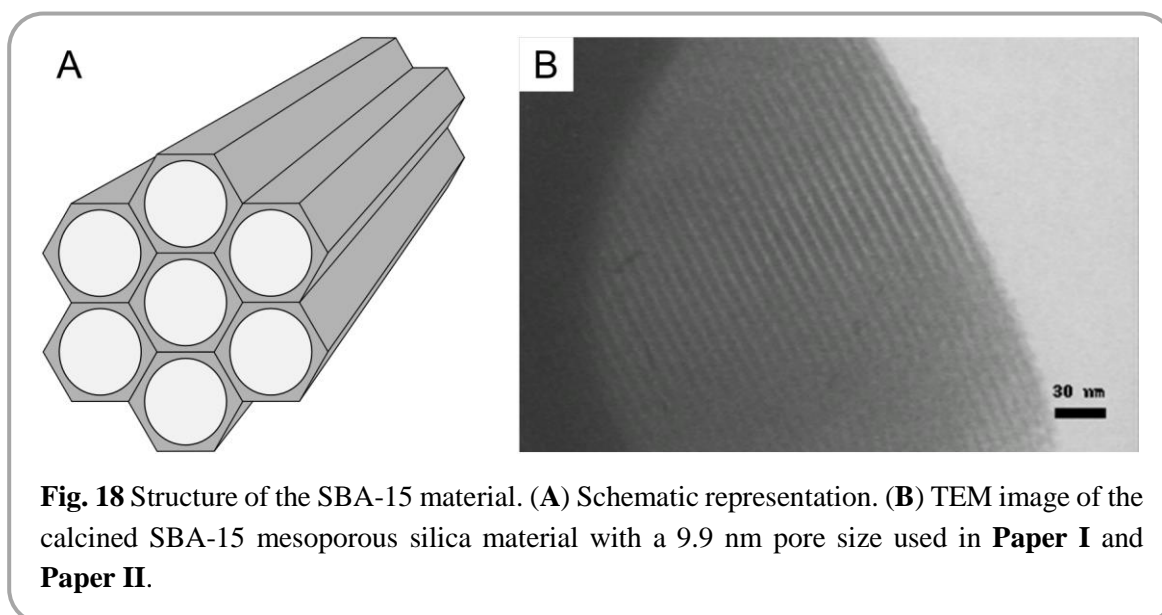
Box 4 Important properties of enzyme immobilization supports

- Inexpensive and environmentally harmless material
- Allows high enzyme loading
- Minimal hydrophobicity (for most enzymes – exception: lipases)
- Density of surface functional-groups (if any), and chemicals required for their activation
- Minimal unspecific interactions between enzyme and support (*e.g.* adsorption) once immobilization step is completed*
- Microbial resistance
- Inert under the operational conditions
- Stable at the process temperature
- Mechanical stability to abrasion (for batch processes) and/or to flow-pressure (for fixed-beds processes)
- Chemical stability towards process chemicals and pH.

*The capacity to make unspecific interactions, which silica possesses, is needed for immobilization by physical adsorption.

6.1 Porous silica supports for enzyme immobilization

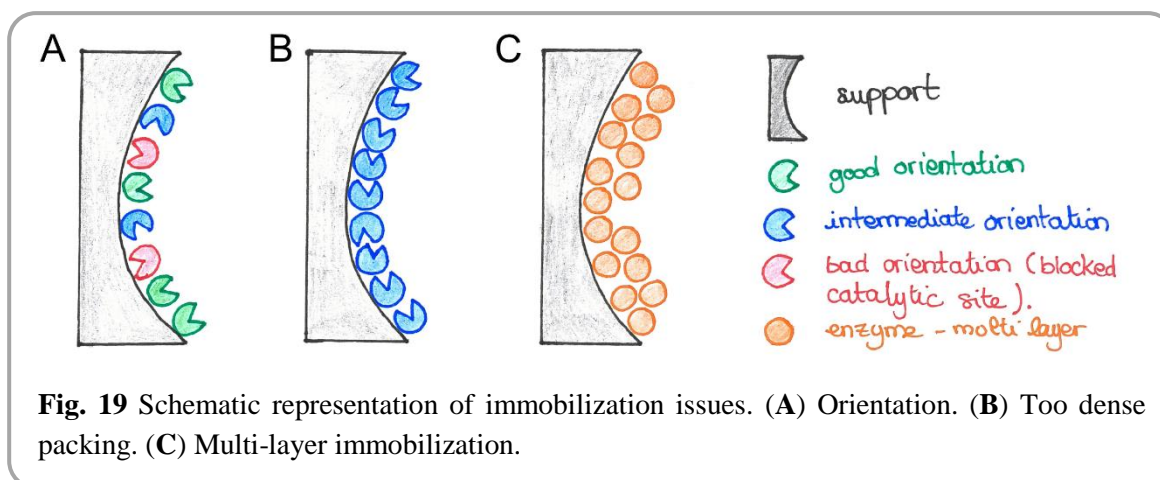
Protocols allowing to control the pore size of silica material through variations in the synthesis conditions have been established over 20 years ago [198]. Developed at the university of California, Santa Barbara (CA, USA), and named after it, the Santa Barbara Amorphous (SBA) materials, are among the most commonly used mesoporous silica (MPS) materials [197]. SBA materials possess an ordered structure which can be cubic (SBA type 11; SBA-11), 3D-hexagonal (SBA-12), 2D-hexagonal (SBA-15, **Fig. 18**), or cubic cage-structured (SBA-16), depending on the template polymers used and synthesis conditions [199]. In **Paper I** and **Paper II**, SBA-15, the most popular SBA material for enzyme immobilization, was used [197]. SBA-15 is a mesoporous material, meaning that its pores have diameters from 2 to 50 nm (comprised between nanopores and macropores) [197,198]. This range of pore diameters have been considered optimal for enzyme immobilization since they are of the same order as the typical diameter of enzymes (as a comparison, the hydrodynamic diameter of the enzyme immobilized in **Paper I** was estimated to be 4 nm) [200]. SBA-15, as other mesoporous materials offers high surface area, typically 500-1400 m².g⁻¹ for SBA-15 [201], which enables high enzyme loading [202]. (The specific area of the support used in **Paper I** and **Paper II** was 439 m².g⁻¹.) Additionally, the silica based SBA-15 also has high chemical and mechanical stability [203], and the silanol groups present at their surface can further be modified and functionalized if desired [197,204–206]. Functionalization can subsequently allow for enzyme immobilization by covalent attachment inside the porous network of the MPS.



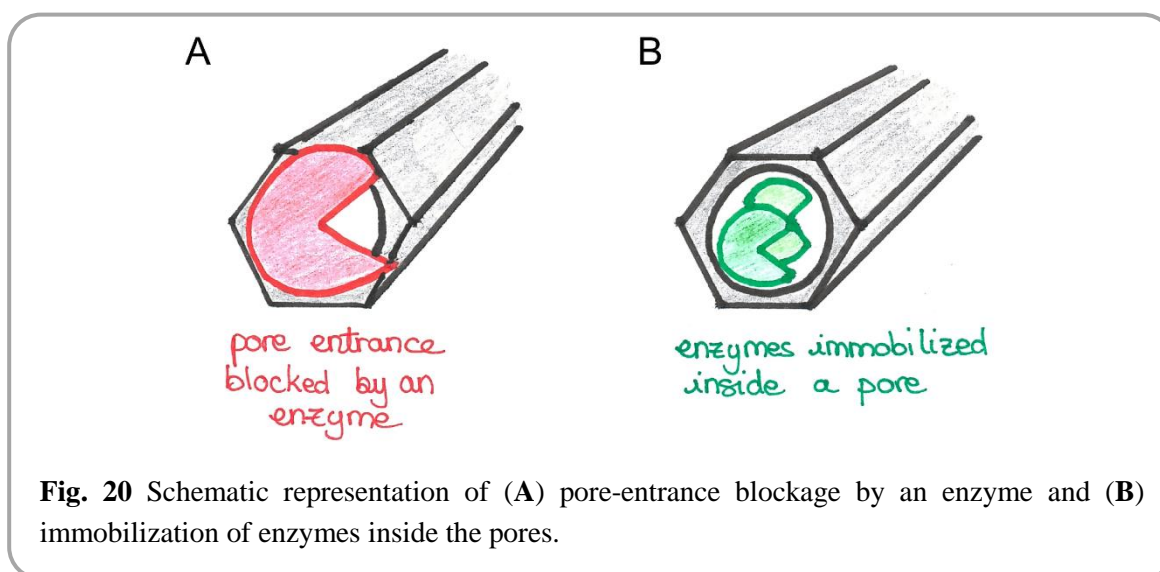
6.2 Influence of MPS properties on immobilization

Increased operational stability has been observed for enzymes immobilized in mesoporous silica materials [165,207,208]. Operational stability can be explained by the fact that when enzymes are immobilized inside the pores they are physically protected from the shearing stress that might arise from the stirring in the reaction vessels or from continuous flow through the reactor [173]. Improved stability has also been reported in the presence of solvents or gases in the reaction medium [201]. It has been hypothesized that the enzymes were protected due to diffusion limitation creating concentration and pH gradients leading to a less toxic micro-environment inside the pores [173,209]. These concentration gradients created by the porous network of MPS can mitigate the effects of too high/low pH values of the bulk solution and of the presence of inhibitors in the reaction mixture [209,210]. Diffusion limitation can also be considered as an advantage in the case of co-immobilization of multiple enzymes able to catalyze cascade reactions (*i.e.* where the product of enzyme A is the substrate of enzyme B) since product/substrate concentration could be locally higher than in the bulk solution [201]. A recent successful example of co-immobilization of two sequentially-acting enzymes in siliceous mesostructured cellular foams resulted in a four times improved specific activity compared to using free enzymes [211].

When immobilized in MPS by physical adsorption, which rely on weak interactions, in principle the enzyme structures should not be distorted. Therefore, other phenomena are likely responsible if decrease or loss of activity is observed. When the orientation of enzymes relative to the support is not controlled during immobilization a common hypothesis for an observed decrease in enzymatic activity is improper orientation of the enzyme [173,212–214]. Indeed, adsorption relies on non-specific interactions and therefore it is not possible to control the orientation of the enzyme on the support. Some of the immobilized enzymes might then be immobilized with the active sites facing the walls of the support (**Fig. 19A**), preventing or limiting interactions with the substrate and subsequent catalytic activity [214]. Steric hindrance can also happen in case of too dense enzyme immobilization in the pores of the support (**Fig. 19B**), or because of immobilization of enzymes on each other, leading to the formation of more than one layer of proteins at the surface of the support (**Fig. 19C**) [215–218]. Both latter effects are linked to high enzyme loadings which result in limited accessibility to the active sites of enzymes for the substrate. For some enzymes decreasing the enzyme loading had beneficial effects on their catalytic performances [219,220].

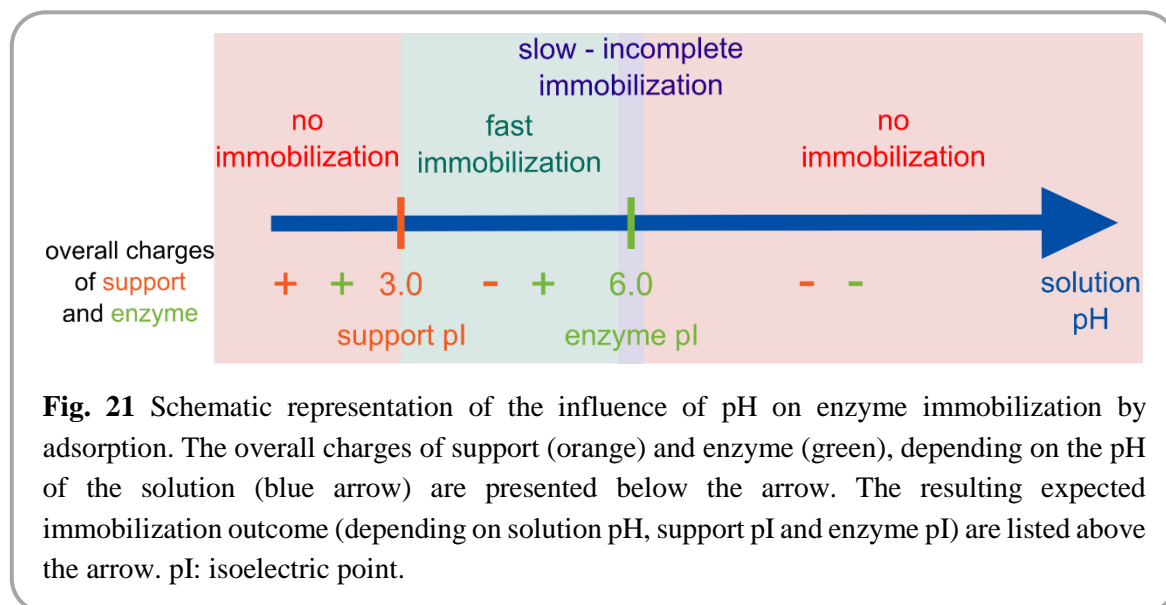


When using MPS as a support, carefully selecting the pore size of the material in relation with the size of the enzyme to be immobilized is also of utmost importance. Selection of a too large pore size could result in enzyme leakage [221], whereas too small pores could prevent the entry of enzymes inside the porous network or result in pores being blocked by enzymes (**Fig. 20**) [215–217,222]. Leakage can be prevented by chemically narrowing the pore diameter after immobilization [221], cross-linking enzymes inside the porous network of the support [223], or using the immobilized enzymes in a non-water environment [170]. The latter relies on hydrophobic interactions which, since most enzymes are hydrophilic molecules, will prevent enzyme leaking into the hydrophobic surrounding solution.



When immobilizing enzymes by physical adsorption, the conditions in which the immobilization is performed, and in particular the pH, greatly influence the outcome. Indeed, electrostatic interactions are a major contributor to enzyme immobilization. The isoelectric point (pI) of SBA-15 is 3.8 [224], which means that when the pH of the bulk solution is higher than 3.8, the support will on average be negatively charged. Enzymes are also charged molecules and their pI value depends on their amino acid composition, but also on which amino acids are located at the surface of the folded enzyme. In order for immobilization to

take place, there should be electrostatic attraction (**Fig. 21**) [176]. Considering the low pI of SBA-15, positively charged enzymes will be best immobilized on this support at physiological pH values.



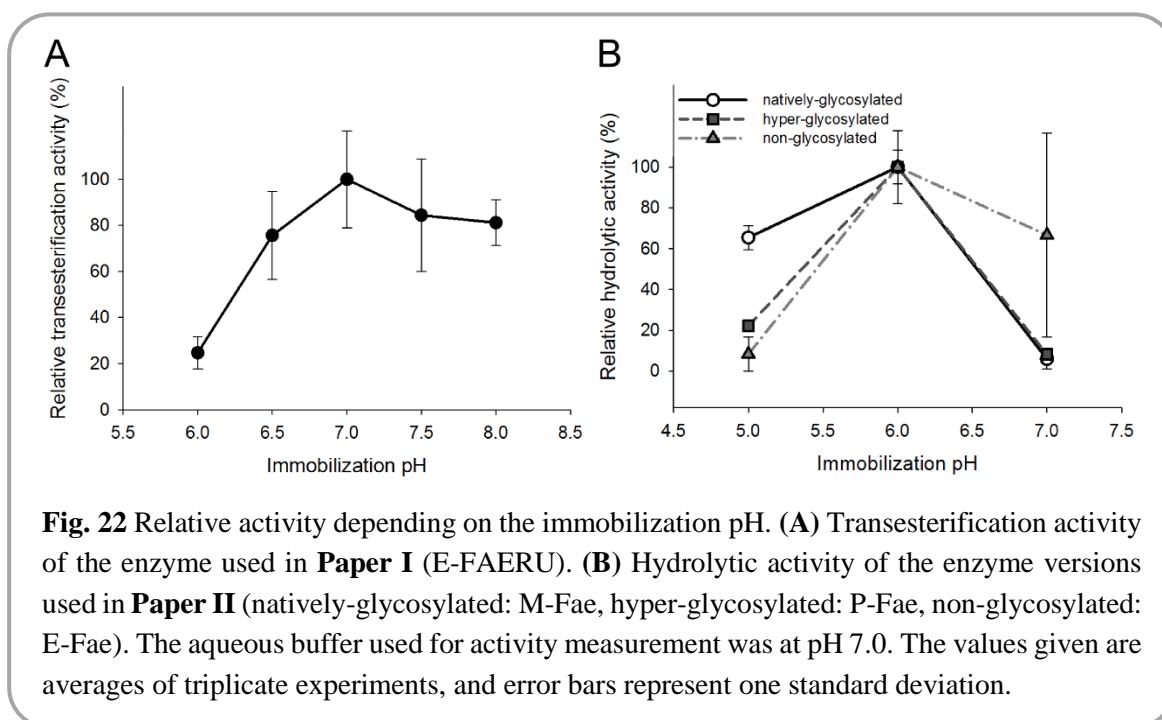
6.3 Influence of glycosylation on enzyme immobilization in MPS

As discussed in **Chapter 4** (section 4.3), glycosylation is important for enzymatic activity. Because the glycan-chains are located at the surface of the folded enzymes, they also influence the apparent hydrodynamic radius of the enzyme [225]. Thus, the presence of large glycan-chains can impede enzyme entrance and diffusion into the pores if they are not taken into account when choosing the MPS pore diameter. In addition, also due to the surface localization of glycan-chains, it has been suggested that glycosylation could alter the apparent pI of proteins through shielding mechanisms [226]. In **Paper II**, comparing the immobilization kinetics of an FAE non-glycosylated, natively-glycosylated, and hyper-glycosylated, we observed three different behaviors. The non-glycosylated enzyme (produced in *E. coli*) was not affected by the pH at which immobilization was performed and was readily immobilized. The natively-glycosylated enzyme (produced in *M. thermophila*) followed the expected pH-dependent immobilization pattern (**Fig. 21**), based on the pI values of the enzyme and support, 6.0 and 3.8, respectively. The hyper-glycosylated enzyme (produced in *P. pastoris*), for which the pI was not experimentally determined, displayed a pH-dependent immobilization pattern that corresponded to an enzyme pI above 6.0. This may be explained by the shielding mechanism mentioned above, leading to an increased pI value. Altered electrostatic interactions between enzyme molecules (*e.g.* less repulsion), also due to glycan-chains shielding, may also have allowed tighter enzyme packing inside the pores. The hyper-glycosylated enzyme was immobilized faster and at higher yields than the natively-glycosylated one. These results demonstrated that the presence of glycan-chains interferes with the immobilization process, probably through more

than one mechanism (*e.g.* size increase, surface shielding), and that the resulting effects could be counter-acting each-other.

6.4 Activity alteration of enzymes immobilized in MPS

The pH at which immobilization is performed impacts immobilization pace and yields (discussed in sections 6.2 and 6.3). It has also been shown that the immobilization pH affects the latter activity of enzymes, irrespective of the pH used during the subsequent catalytic reaction [227] (**Paper I**, **Paper II**). Thörn *et al.* proposed the hypothesis of a “memory effect” to explain this phenomenon: the pH used during immobilization alters the enzyme structure configuration and these changes, due to immobilization, remain even if the solution pH value is subsequently changed [227]. In **Paper I** and **Paper II**, I observed up to 90% variations in the specific activity levels depending on the immobilization pH (all activity tests were run at a reaction pH value of 7.0) when studying transesterification (**Paper I**, **Fig. 22A**) as well as hydrolysis (**Paper II**, **Fig. 22B**). When comparing the activity levels of the natively-glycosylated enzyme used in **Paper II** before and after immobilization, the activity of this enzyme version was less adversely affected than its hyper-glycosylated and non-glycosylated counterparts. These results suggest that the native glycosylation pattern may have protected it from structural changes during immobilization.



In addition, as was observed with other types of immobilization techniques, some enzymes immobilized in MPS had altered substrate selectivity and/or reaction specificity [173,209,228]. Reaction specificity alteration is especially interesting when using hydrolytic enzymes to perform synthetic reactions, as discussed in **Chapter 2** (section 2.5), since it may contribute to favor synthesis over hydrolysis. It has been demonstrated for an FAE that its activity was shifted towards transesterification when immobilized in SBA-15 [228]. This

behavior appears to be enzyme-dependent, as Hüttner *et. al.* found an increased selectivity for transesterification after immobilization only for one of the four FAEs [229]. For the FAE I immobilized in **Paper I**, hydrolysis remained the dominant activity in all tested conditions. Moreover, the kinetic parameters determination performed before and after immobilization on this enzyme, revealed that the Michaelis constant, K_m , was similar whether the enzyme was free or immobilized. The decreased catalytic activity was a result of a strong decrease in the enzyme turnover number (k_{cat} ; 4.8-fold and 14-fold decrease for hydrolysis and transesterification, respectively).

No temperature stabilization was obtained by immobilizing any of the FAEs used in **Paper I** and **Paper II**, probably due to the immobilization technique used, physical adsorption, which usually does not confer extra structural stability to the enzymes. However, in **Paper I**, the reusability test carried out for 10 cycles of 48 h each in butanol (92.5%) demonstrated that the immobilized enzyme retained 44% and 26% of its initial hydrolytic and transesterification activities, respectively. Hence, after immobilization, the enzyme could be used in successive reaction-batches for 20 days, emphasizing the potential of enzyme immobilization for industrial applications.

7. Conclusion

During my thesis project, I studied several esterases, fungal and bacterial, originating from microorganisms found in diverse ecological niches and evaluated their biochemical properties. The impact of *N*-glycosylation on catalytic activity and immobilization was investigated on one FAE and the transesterification capabilities before and after immobilization were tested on another FAE. The work presented in my thesis provides new knowledge on feruloyl esterases and factors influencing their performance (*e.g.* production host, immobilization, synthetic reactions potential). Increasing knowledge about feruloyl esterases, and about esterases in general, is important to better understand the fundamental mechanisms of these enzymes, their role in conversion of plant biomass as well as their potential for various industrial applications.

Functional annotations were used to guide the selection of bacterial (**Paper III**) and fungal (**Paper IV**) targets with putative FAE activity. Annotations proved correct in identifying seven actual enzymes which all possessed esterase activity. Among the five enzyme targets studied in **Paper IV**, only one enzyme was not confirmed as a tannase or feruloyl esterase. This might be linked to the use of synthetic substrates for enzyme characterization. For the same reason, a doubt remains regarding whether the CE1 module from the *B. ovatus* enzyme has FAE activity or not (**Paper III**). These results exemplify that functional annotations can be of great help in enzyme discovery but also emphasize the need for experimental activity confirmation of the predictions.

The two bacterial enzymes studied in **Paper III** were both multi-catalytic esterases found in PULs. The catalytic activity of the *B. ovatus* enzyme was better when the two modules were physically linked than when equivalent amounts of the two domains were separately added. The activity improvements observed with the *B. ovatus* enzyme could arise from physical proximity of the catalytic modules, as observed with cellulosomes [38,39], or could be due to improved timing in the catalysis (*i.e.* simultaneous or coordinated sequential action of the two esterases). In the assay conditions tested, and on the substrates used, no beneficial effect of producing the two catalytic modules physically linked together were observed for the *F. johnsoniae* enzyme. However, in its natural reaction environment, the *F. johnsoniae*

enzyme might also be a more efficient catalyst when its two CE modules are linked. These results, in addition to providing the *in vitro* biochemical characterization of two novel multi-catalytic esterases, point out the difficulty and caution one should keep when drawing conclusions on the activity and role of enzymes in their native environment based on *in vitro* activity measurements using synthetic substrates.

Most of the esterases used in my work were obtained through heterologous protein production. **Paper II** and **Paper IV** demonstrated that the host selection for heterologous production must be done carefully. Indeed, producing fungal enzymes in the bacterium *E. coli*, even with folding help from chaperones, resulted in very low yields of functional enzyme (if any). Production in the methylotrophic yeast, *P. pastoris*, was challenging as low enzyme yields or instable enzymes were obtained for most of the fungal enzyme targets (**Paper IV**), demonstrating that even in well-known microbial hosts heterologous protein production is not yet straightforward or predictable.

Remarkably, I could demonstrate the drastic impact that *N*-glycosylation can have on an enzyme final characteristics by studying different versions of an FAE produced in different microorganisms (the three versions had the same amino acid chain; **Paper II**). The impact of the lack of glycosylation (as observed for the version produced by *E. coli*) was known and expected [147,156]. Interestingly, substantial differences were also observed between the two glycosylated enzyme versions. The glycan-chains present on these two enzymes version proved different when analyzed by mass spectrometry and I believe the reason (or at least a major contributor) for the differences observed during biochemical characterization were these differences in glycan-chain length. The most striking difference between the glycosylated enzyme versions was temperature stability. I found a 10°C higher optimal temperature for activity with the enzyme version homologously produced in *M. thermophila* compared to the enzyme version heterologously produced in *P. pastoris*. **Paper II** demonstrated the importance of considering the glycosylation pattern of the production host and underlined the fact that obtaining an active enzyme by heterologous production does not mean that the enzyme will possess its native characteristics. Taken together, the results from **Paper II** and **Paper IV** advocate for a careful selection of enzyme production hosts.

Immobilization in mesoporous silica (SBA-15) by physical adsorption was successfully achieved for the FAE studied in **Paper I** as well as for the three enzyme versions studied in **Paper II**. Yields and rates of immobilization depended on the pH of the solution during immobilization and on the properties of enzymes. The results presented in **Paper II** demonstrated that the presence of glycan-chains on the protein influenced the immobilization kinetics, and that the resulting effect was a combination of modifications of size and surface properties.

After immobilization, the catalytic performances of the immobilized enzymes were decreased compared to that of their corresponding free enzymes in both studies (**Paper I** and **Paper II**). Kinetic parameter determination of the FAE studied in **Paper I**, before and after

immobilization, demonstrated that the decrease in catalytic efficiency observed for this enzyme (both in hydrolysis and transesterification) was due to reductions in the turnover number (k_{cat}) since K_m was not affected. The catalytic activity of this commercial FAE was strongly hydrolytic, and unlike previous studies reported, hydrolysis remained its dominant activity even after immobilization. The enzyme however exhibited a good reusability in a 92.5% butanol reaction system, suggesting that it is a good candidate for immobilization, but that optimization of the immobilization conditions, technique and support chosen must be done, especially if transesterification is the desired reaction.

Overall, one common feature of the results obtained in the four studies in my thesis is the demonstration that results in the esterase field are most of the time enzyme-dependent. Meaning that one cannot infer the behavior of an FAE in terms of hydrolytic activity, substrate selectivity, capacity to perform transesterification, and properties after immobilization before testing this particular FAE. Hence, more data and knowledge, especially on sparsely studied enzymes such as FAEs, are needed in order to render patterns and trends visible. This in turn will help refine prediction algorithms and will contribute to guide enzyme discovery, enzyme selection for a specific application, and enzyme immobilization more efficiently. The final outcome being that the more knowledge we have about the enzymes or enzymatic systems we utilize, the easier, cheaper and reliable industrial applications will become.

8. Future perspectives

Currently, identifying the most suitable FAE, host for heterologous production of that enzyme, or immobilization support and technique to create a robust immobilized enzyme that suits a specific application is based on a trial and error approach. In order to render this selection less empirical and to decrease the amount of potential combinations (*i.e.* enzyme – production host – immobilization support) to test, more knowledge and understanding of the characteristics of enzymes and/or of their interactions with immobilization supports are needed. Solving protein structures of more FAEs would increase our knowledge of these enzymes and of their structure function relationship. This information might enable the detection of structural features, and amino acid sequences, which are specific to FAEs, thereby allowing some refining of the existing functional annotation algorithms. Getting access to more structures of FAEs in the presence of a substrate or ligand would deepen our understanding of the mechanisms of recognition, binding and hydrolysis of these enzymes. Recently, Gruninger *et. al.* presented the first structure of a fungal FAE possessing a β -clamp [230], a structural feature that proved essential for the activity of this enzyme. The presence of this mobile β -clamp that interacts with the substrate and closes the active site of the enzyme also sterically prevents the binding of di-ferulate structures. Mapping such structural features that are crucial for the activity and substrate selectivity of FAEs could help classifying these enzymes, designing efficient hemicellulolytic cocktails and guiding the selection of enzymes for synthetic reactions or immobilization.

The substrate(s) used to test an enzymatic activity is always an important factor to consider. Recently, the characterization of FAEs were based on a limited set of synthetic substrates: the four methyl-esters presented in **Fig. 6** as well as 4-nitrophenyl ferulate. None of these substrates present di-ferulate structures, nor do they accurately represent the bulky substrates that FAEs encounter when acting on plant biomass. Therefore, continued efforts are needed to test and compare the catalytic activity of FAEs on synthetic substrates to their activity on polysaccharides substrates obtained from biomass. Concurrently, the gap between the synthetic substrates routinely used for activity measurements and the substrates that enzymes were evolved to act on need to be reduced in order to reduce the risks of wrongly assigning the activity type of enzymes or even failing to identify some proteins as enzymes.

The findings I presented in **Paper II** demonstrated the drastic impact of the glycan-chains length on the characteristics of an FAE, especially on its thermostability. Further research should be conducted to determine whether this FAE was an isolated case or if other enzymes are also greatly affected by glycan-chains length and composition. If more FAEs and enzymes were shown to have changed properties depending on the length and composition of the glycan-chains they carry, four major questions would need to be addressed: (i) Is there any optimal chain-length or composition? (ii) What are the typical glycosylation patterns of the native microorganisms of commercially relevant enzymes? (iii) Could glycan engineering be used instead of, or in complement to, enzyme engineering to improve the thermostability or other desired properties of enzymes? (iv) Are there other post-translational modifications that do affect enzyme properties as *N*-glycosylation does? In the future, similar to what has been performed to engineer microbial strains that produce human-like glycans [142], production hosts mimicking the glycosylation pathways of other microorganisms might become available. Current efforts to discover new microbes and to establish novel microbial production hosts should also be pursued.

Structural information about FAEs would also be an invaluable asset for understanding the synthetic capabilities of these enzymes. Indeed, Antonopoulou *et. al.* recently demonstrated that the selectivity of FAEs for transesterification was linked to structural features of the enzymes studied, such as binding cavity size and accessibility or surface properties of the enzymes around the active site [53]. In addition, they clustered enzymes based on synthetic capabilities, which revealed that some of the groups were similar to the ones presented in the phylogenetic subfamily classification recently published by Dilokpimol *et. al.* [231]. Both of these studies only investigated fungal FAEs. Therefore, future research should aim at evaluating the synthetic potential of bacterial FAEs and at including protein structural information when possible in order to increase our understanding of FAEs esterification and transesterification reaction mechanisms.

Finally, protein structural information is also needed in order to understand and explain the changes in behavior observed on enzymes after immobilization. This is of utmost importance in the case of immobilization techniques which rely on the surface properties of the enzymes, such as physical adsorption. Indeed, through structure determination, scientists can get information about the surface exposed residues and thereby to the overall surface charges and hydrophilicity of the enzymes. Precise knowledge about enzyme surface properties might enable better predictions of the predominant forces and interactions driving the immobilization as well as the orientation of the immobilized enzymes relative to the support. These interaction prediction might further enable us to choose or chemically modify the surface of immobilization support in order to strengthen the desired properties of an immobilized enzyme (e.g. substrate selectivity, reaction specificity). Structural knowledge is also needed to perform *in silico* molecular dynamic simulations. These simulations may provide atomic-level structural and dynamic information on the interactions of the enzymes with their substrates and on their conformational changes during catalysis. Such information

should be sought in order to understand the activity and stability changes that can be observed after immobilization of an enzyme.

Increasing our fundamental knowledge of feruloyl esterases, other esterases, and enzymes in general will allow us to optimize every step from enzyme discovery, to production and immobilization. Which will overall decrease the costs of enzyme-based processes and should increase and improve our utilization of enzymes in industrial settings. While increasing fundamental knowledge, we may also discover new enzymatic activities or novel applications for enzymes, expanding further the possible applications of these powerful proteins.

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